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Identification of novel protein kinase regulators of TGFβ signalling

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Identification of novel protein kinase regulators of TGFβ signalling

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DECLARATIONS

I declare that the following thesis is based upon experimental investigations conducted by myself, and that this thesis is of my own composition. Research other than my own is indicated by reference to the relevant publications or researchers. Furthermore, this dissertation has not previously been submitted for a higher degree.

Luke Daniel Hutchinson

I certify that Luke Daniel Hutchinson has completed the equivalent of at least nine terms in research work in the Medical Research Council (MRC) Protein Phosphorylation and Ubiquitylation Unit within the School of Life Sciences at the University of Dundee and that he has fulfilled the conditions of the Ordinance General No. 14 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy (PhD).

Dr. Gopal P. Sapkota

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THESIS SUMMARY

The signalling pathways initiated by members of the transforming growth factor-β (TGFβ) family of cytokines perform critical functions during embryonic development and adult tissue homeostasis. Accordingly, TGFβ family members are involved in the regulation of a diverse array of cellular processes including epithelial-mesenchymal transition (EMT), differentiation, cellular proliferation, extracellular matrix (ECM) production and apoptotic cell death. Hence, genetic malfunctions in the TGFβ-induced signalling pathways are implicated in the pathogenesis of multiple somatic and inherited human pathologies, most notably fibrosis and tumourigenesis and metastasis. Accumulating evidence has illuminated the context-dependent nature of TGFβ signalling and provided a molecular framework by which to explain how a conceptually simple signal transduction pathway is able to control diverse cellular responses. Thus, further elucidation of the molecular basis underlying the contextual determinants of TGFβ signalling will evidently provide a greater understanding of how TGFβ signalling operates in different cellular contexts and inform research focused on pharmacologically targeting the pathway effectively.

This thesis project began by conducting a pharmacological screen using an endogenous TGFβ-dependent transcriptional reporter cell line in order to identify potential novel regulatory components of the TGFβ signalling pathway. From this screen and subsequent validation experiments, I observed that three structurally unrelated small-molecule inhibitors that target the salt-inducible kinases (SIKs) are able to attenuate the TGFβ-mediated transcriptional regulation of specific target genes. Moreover, this transcriptional attenuation occurred without affecting the receptor-mediated phosphorylation of the R-SMAD transcription factors SMAD2 or SMAD3 nor the ability of activated SMAD transcriptional complexes to translocate into the nucleus.

The SIKs, of which there are three isoforms, are serine-threonine specific protein kinases that belong to the AMPK-related family of kinases. To date, the reported cellular functions of SIK isoforms are primarily in the control of innate immune signalling and metabolic regulation. The SIKs primarily function by phosphorylating and hence regulating the activity of the transcriptional coregulators CREB-regulated transcriptional coactivators (CRTCs) and the class IIa histone deacetylases (HDACs) HDAC4 and HDAC5. From the research conducted during this thesis project, it is now evident that SIKs also function to modulate

TGFβ-dependent transcriptional responses. However, the precise molecular mechanisms underlying this observation remain to be elucidated.

In addition to small-molecule kinase inhibition of SIK isoforms, I have demonstrated that genetic inactivation of SIK2 and SIK3 results in the attenuation of TGFβ-dependent transcriptional induction of the target gene PAI-1. Moreover, both PAI-1 transcript and protein expression are attenuated in cells which are deficient in the endogenous expression of LKB1, the upstream activating kinase of SIK isoforms. Crucially, restoration of wild type, but not kinase inactive LKB1, potentiates both basal and TGFβ-induced PAI-1 expression. The effect of small-molecule SIK inhibitors was also investigated in the context of the TGFβ-dependent cellular proliferation and apoptotic responses. Inhibition of SIKs was sufficient to enhance the anti-proliferative response in certain cell lines. Furthermore, treatment of cells with small-molecule SIK inhibitors potentiated TGFβ-mediated apoptotic cell death. Collectively, these findings not only identify SIK isoforms as novel regulators of TGFβ signalling but also imply that inhibiting SIKs might sensitise certain cancers for TGFβ-mediated apoptosis.

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ABBREVIATIONS

°C	degree Celsius
2-ME	2-Mercaptoethanol
2G	second-generation
4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
ABB	Annexin Binding Buffer
ABL	Abelson murine leukaemia viral oncogene homolog
ACVR	activin receptor
AD	autosomal dominant
ADP	adenosine-5'-diphosphate
AKT	RAC-alpha serine/threonine-protein kinase
ALK	activin receptor-like kinase
ALL	acute lymphoblastic leukaemia
AMD	auto-modification domain
AMH	anti-Muellerian hormone
AML	acute monocytic leukaemia
AMP	adenosine-5'-monophosphate
AMPK	5'-AMP-activated protein kinase
ANOVA	analysis of variance
aPKC	atypical protein kinase C
APS	ammonium persulfate
ARE	activin-responsive element
ATF-2	cyclic AMP-dependent transcription factor 2
ATF3	activating transcription factor 3
ATP	adenosine-5'-triphosphate
BAD	BCL2-associated agonist of cell death
BAK	BCL-2 homologous antagonist/killer
BCL-2	B-cell lymphoma 2
BH	BCL-2 homology
bHLH	basic helix-loop-helix

BID	BH3-interacting domain death agonist
BIK	BCL2-interacting killer
BIM	BCL2-interacting mediator of cell death
BL	Burkitt lymphoma
BMDC	bone-marrow-derived dendritic cells
BMF	BCL2-modifying factor
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BOK	BCL-2-related ovarian killer protein
bp	base pair
BRCA	breast cancer type susceptibility protein
BRE	BMP responsive elements
BRSK	brain-specific serine/threonine-kinase
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
C	cytoplasmic
C-terminal	carboxy-terminal
Ca^{2+}/CaM	calcium/calmodulin
CAMK2	calcium/calmodulin-dependent protein kinase 2
cAMP	cyclic adenosine monophosphate
Cas9	CRISPR-associated protein 9
CCLR	Cell Culture Lysis Reagent
$CD4^+$	T-cell surface glycoprotein CD4
CDC42	cell division control protein 42 homolog
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CIP/KIP	CDK interacting protein/kinase inhibitory protein
CK1γ2	casein kinase 1 isoform gamma-2
cpm	counts per minute
CREB	cAMP-responsive element-binding protein

CRISPR	clustered regularly interspaced short palindromic repeats
CRTC	CREB-regulated transcriptional co-activator
CTGF	connective tissue growth factor
Da	dalton
DAPI	4’,6-diamidino-2-phenylindole
DAPK1	death-associated protein kinase 1
DAP6	death domain-associated protein 6
DBD	DNA-binding domain
DDR2	discoidin domain-containing receptor 2
DMEM	Dulbecco’s Modified Eagle’s Medium
DMP	dimethyl pimelimidate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco’s Phosphate-Buffered Saline
DSB	DNA double-stranded break
DTT	dithiothreitol
DUB	deubiquitylating
E-box	enhancer box
ECL	Enhanced Chemiluminescent
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis (2-aminoethyl ether)-*N*,*N*,*N*′,*N*′,tetraacetic acid
EMA	European Medicines Agency
EMT	epithelial-mesenchymal transition
EP300	E1A-associated protein p300
EPHB4	ephrin type-B receptor 4
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cell
FBS	fetal bovine serum
FGF	fibroblast growth factor

FGFR	fibroblast growth factor receptor
FKBP12	FK506-binding protein 12
FOXH1	forkhead box protein H1
FOXO3	forkhead box protein O3
FOXP3	forkhead box protein P3
FSC	forward light scatter
g	gram or gravity
GADD34	growth arrest and DNA damage protein 34
GADD45B	growth arrest and DNA damage-inducible protein 45
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDF	growth and differentiation factors
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G-protein-coupled receptors
GRB2	growth factor receptor-bound protein 2
GRK2	G-protein coupled receptor kinase 2
gRNA	guide ribonucleic acid
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
GTP	guanosine-5’-triphosphate
GWAS	genome-wide association studies
HAT	histone acetyltransferase
HCl	hydrochloric acid
HDAC	histone deacetylase
HECT	homologous to E6-AP carboxyl terminus
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	homologous recombination
HRK	activator of apoptosis harakiri
HRP	horseradish peroxidase
HRV	human rhinovirus

HTS	high-throughput screening
I-SMAD	inhibitory SMAD
IBD	inflammatory bowel disease
IC_{50}	half maximal inhibitory concentration
ID-1	DNA-binding protein inhibitor ID-1
IGF	insulin-like growth factor
IgG	immunoglobulin G
IL	interleukin
INK4	inhibitors of CDK4
IP	immunoprecipitation
IRES	internal ribosome entry site
JAMM	JAB1/MPN/MOV34
JNK	c-Jun amino-terminal kinase
KA	keratoacanthomas
kDa	kilodalton
KI	knock-in
L	litre
LAP	latency-associated peptide
LDS	lithium dodecyl sulfate
LDTF	lineage-determining transcription factors
LEFTY	left-right determination factor
LKB1	liver kinase B1
LOH	loss of heterozygosity
LTBP	latent TGFβ-binding protein
m	milli- or metre
M	Molar concentration or mitotic phase
MAD4	MAX dimerisation protein 4
MAPK	mitogen-activated protein kinase
MARK	MAP/microtubule affinity-regulating kinase
MAX	MYC-associated factor X
MBP	maltose-binding protein
MCL1	induced myeloid leukaemia cell differentiation protein

MEF	mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MELK	Maternal embryonic leucine zipper kinase
MEM	Minimum Essential Medium
$MgCl_2$	magnesium chloride
MH1	MAD homology 1
MH2	MAD homology 2
MINDY	MIU-containing novel DUB family
MIU	motif interacting with ubiquitin
MJD	Machado-Joseph disease
MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MMP	matrix metalloproteinase
mol	mole
MOPS	3-Morpholinopropane-1-sulfonic acid
MPK38	murine protein serine-threonine kinase 38
MRC PPU	Medical Research Council Protein Phosphorylation and Ubiquitylation Unit
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
MW	molecular weight
MXI-1	MAX-interacting protein 1
n	nano- or number
N	nuclear
N-terminal	amino-terminal
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
NEAA	Non-Essential Amino Acids
NEDD4	neural precursor cell expressed developmentally down-regulated protein 4
NFAT	nuclear factor of activated T-cells
NGF	nerve growth factor
NLS	nuclear localisation signal

NP-40	Nonidet P-40
NSCLC	non-small cell lung carcinoma
NUAK	NUAK family SNF1-like kinase
OD	optical density
OTU	ovarian tumour protease
OTUB1	OTU domain-containing ubiquitin aldehyde-binding protein 1
pADPr	poly-ADP-ribosylate
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PAR6	partitioning defective 6 homolog alpha
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCSK6	proprotein convertase subtilisin/kexin type 6
PDGF	platelet-derived growth factor
PEI	polyethylenimine
Ph+	Philadelphia chromosome-positive
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PJS	Peutz-Jeghers syndrome
PK	pharmacokinetic
PKB	protein kinase B
PP1	serine/threonine-protein phosphatase PP1
PP5	serine/threonine-specific protein phosphatase 5
PPM1A	protein phosphatase 1A
PS	phosphatidylserine
PTK6	protein-tyrosine kinase 6
PTM	post-translational modification
PUMA	p53 upregulated modulator of apoptosis
R-point	restriction point
R-SMAD	receptor-regulated SMAD
RAPTOR	regulatory-associated protein of mTOR
RBL1	retinoblastoma-like protein 1

RICTOR	rapamycin-insensitive companion of mTOR
RNA	ribonucleic acid
RNA-Seq	ribonucleic acid sequencing
RNAi	ribonucleic interference
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RTK	receptor tyrosine kinases
RUNX2	runt-related transcription factor 2
S6K1	ribosomal protein S6 kinase beta-1
SARA	SMAD anchor for receptor activation
SBD	SMAD-binding domain
SBE	SMAD binding element
SCC	squamous cell carcinoma
SCF	Skp1, Cullin, F-box containing complex
SCP	small carboxy-terminal domain phosphatases
SD	standard deviation
SDS	sodium dodecyl sulfate
SDTF	signal-driven transcription factors
SEM	standard error of the mean
sgRNA	single-guide ribonucleic acid
shRNA	short hairpin ribonucleic acid
SIK	salt-inducible kinase
siRNA	small interfering RNA
SMAD	Sma/mothers against decapentaplegic
SMI	small-molecule inhibitor
SMURF	SMAD ubiquitylation regulatory factor
SNP	single-nucleotide polymorphisms
SNRK	sucrose non-fermenting related kinase
SOS	son of Sevenless
Src	sarcoma
SSC	side light scatter
STRAD	STE20-related kinase adaptor protein
SV-40	Simian virus 40

T-loop	activation loop
t-PA	tissue-type plasminogen activator
TAK1	TGFβ-activated kinase 1
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing TWEEN 20
TEMED	tetramethylethylenediamine
TGFβ	transforming growth factor-beta
TGFβR	transforming growth factor-beta receptor
TIE	TGFβ inhibitory element
TIEG1	TGFβ-inducible early-response gene
TKI	tyrosine kinase inhibitor
TKL	tyrosine kinase-like
TLR	Toll-like receptors
TM	transmembrane
TNF	tumour necrosis factor
TP53	cellular tumour antigen p53
TRAF6	TNF receptor-associated factor 6
T_{reg}	regulatory T cell
Tris	tris(hydroxymethyl)methylamine
u-PA	urokinase-type plasminogen activator
Ub	ubiquitin
UC	ulcerative colitis
UCH	ubiquitin carboxy-terminal hydrolase
US FDA	United States Food and Drug Administration
USP	ubiquitin-specific proteases
V	volt
v/v	volume to volume ratio
VEGF	vascular endothelial growth factor
w/v	weight to volume ratio
WT	wild type
WW	tryptophan-tryptophan
ZEB	zinc finger E-box-binding homeobox

ZO-1	zonula occludens-1
µ	micro-

AMINO ACID CODE

Amino acid	Three-letter code	Single-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid	Xaa	X

PUBLICATIONS

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1 INTRODUCTION

1.1 TRANSFORMING GROWTH FACTOR-β (TGFβ) SIGNALLING

The signalling pathways initiated by the transforming growth factor-β (TGFβ) family of cytokines are amongst the most prevalent and diverse in metazoan biology. TGFβ cytokines are known to regulate a multitude of cellular processes including cell proliferation and differentiation, epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) production, immune regulation and apoptosis, in a context-dependent manner. Therefore, perturbations in the signalling pathway have been implicated in the development of many pathological conditions such as tumorigenesis and metastasis, fibrotic disorders, congenital diseases and dysregulation of the immune system (Elliott and Blobe, 2005; Gordon and Blobe, 2008).

1.1.1 The TGFβ cytokines

The TGFβ family of structurally related cytokines are encoded by 33 genes in the human genome and includes the three prototypical TGFβ members, Nodal, left-right determination factor 1 and 2 (LEFTY1 and LEFTY2), inhibin-α, four inhibin-β chains (heterodimers of which form activin isoforms), bone morphogenetic proteins (BMPs), anti-Muellerian hormone (AMH, alternatively referred to as Muellerian-inhibiting substance; MIS) and growth and differentiation factors (GDFs). These members can be further divided into sub-families; the TGFβ/activin/nodal subfamily and the BMP/GDF/MIS subfamily. This subdivision is based on a number of structural and functional considerations including sequence similarity, preferences for different cell surface receptors and differences in downstream SMAD activation (Shi and Massagué, 2003).

All of the 33 genes for TGFβ and BMP cytokines encode large precursor polypeptides with similar sequence organisation, consisting of an amino-terminal secretion signal peptide, a large, approximately 250 residue precursor segment (termed prodomain or prosegment) and a carboxy-terminal polypeptide that confers the biological activity of the mature dimeric

form of the protein. The amino-terminal secretion signal peptide is cleaved during translocation of the polypeptide into the lumen of the rough endoplasmic reticulum (RER) (Morikawa, Derynck and Miyazono, 2016). The amino acid sequence of the carboxy-terminal polypeptide defines the structural similarity of TGFβ cytokines, whereas the sequence of the prodomain polypeptide is divergent amongst the family members, varying in both length and sequence homology (Derynck *et al.*, 1988). In order to generate the biologically active signalling proteins, the inactive precursor polypeptides of TGFβ/BMP members undergo proteolytic processing. For example, the 55 kDa pro-TGFβ1 polypeptide requires endoproteolytic cleavage conducted by the serine proprotein convertase furin, resulting in the separation of the 44 kDa prodomain from the 12.5 kDa mature TGFβ1 monomer (Dubois *et al.*, 1995). Similarly, the precursor polypeptides of Nodal and BMP4 can be proteolytically processed by either furin or another proprotein convertase termed PACE4 (alternatively referred to as proprotein convertase subtilisin/kexin type 6; PCSK6) (Constam and Robertson, 1999). The importance of this endoproteolytic regulation is highlighted by the dysregulation of anteroposterior and left-right axis formation that occurs during early embryonic development in PACE4-deficient mouse embryos (Constam and Robertson, 2000). Interestingly, TGFβ isoforms are secreted from cells in a biologically inactive form termed latent TGFβ, which consists of the mature TGFβ homodimer non-covalently associated with the previously cleaved prodomain polypeptide. In this context, the prodomain is subsequently referred to as the latency-associated peptide (LAP) (Miyazono *et al.*, 1988; Wakefield *et al.*, 1988). The LAP protein is itself dimeric, resulting from the formation of intermolecular disulphide bonds between monomers, and functions to prevent the mature TGFβ homodimer from interacting with cognate cell surface receptors. This latent TGFβ complex is further associated with latent TGFβ-binding proteins (LTBPs) which assist with correct complex formation and may define the extracellular localisation of the latency complex (Miyazono *et al.*, 1991). The liberation of the active TGFβ cytokine from the latency complex is therefore an important regulatory event in the TGFβ signalling pathway and can be achieved through a variety of mechanisms. A number of different proteases have been reported to be involved in the proteolytic activation of latent TGFβ including the serine protease plasmin (Lyons, Keski-Oja and Moses, 1988) and matrix metalloprotease-9 (MMP-9) (Yu and Stamenkovic, 2000).

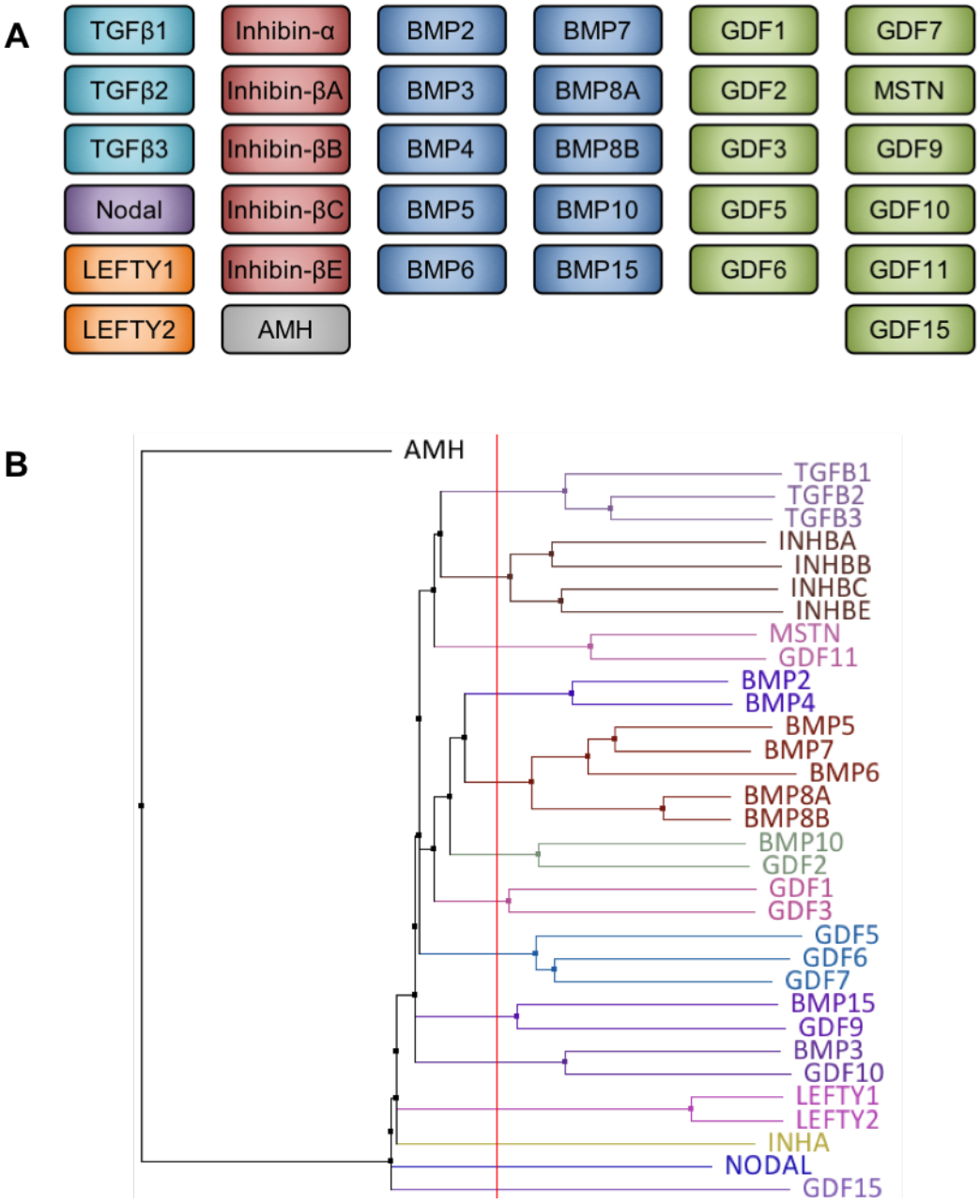
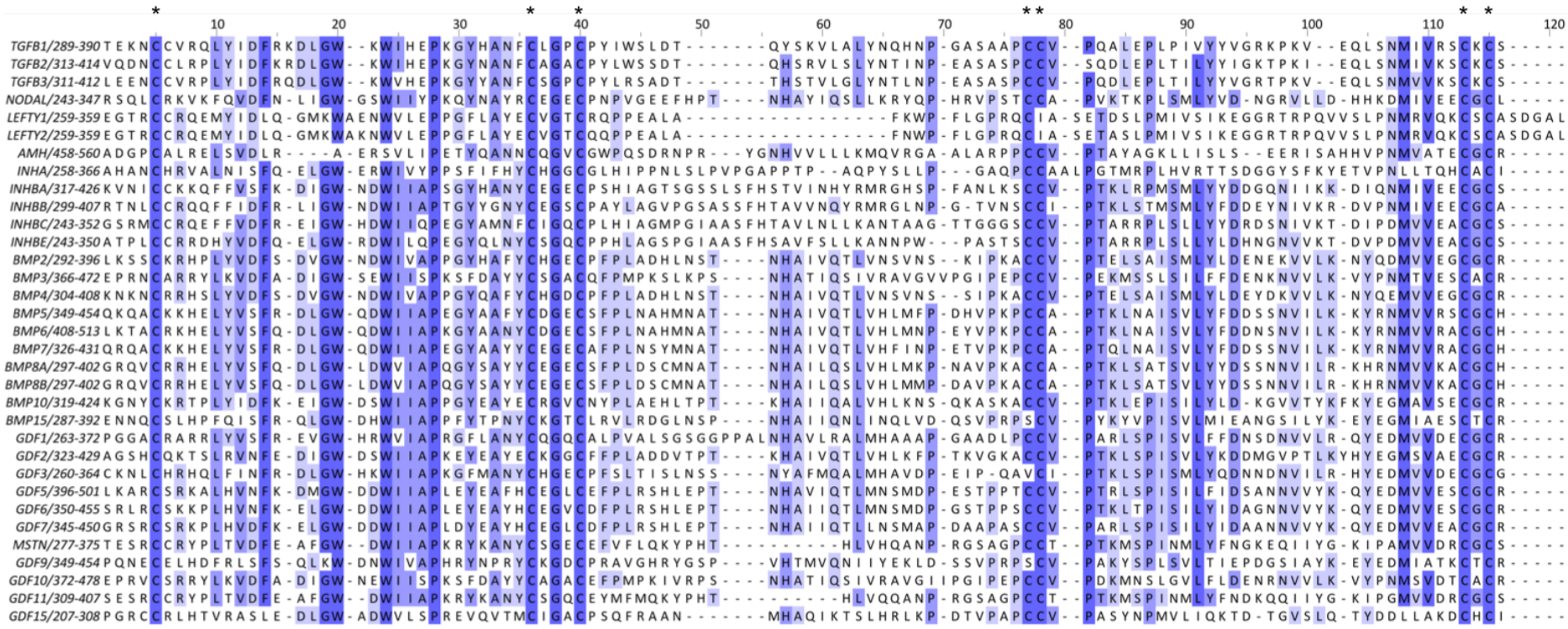


Figure 1A. The TGFβ family of cytokines

(A) The human genome encodes 33 genes of the transforming growth factor β (TGFβ) family of cytokines including 3 TGFβ isoforms, 4 inhibin-β subunits, 10 bone morphogenetic proteins (BMPs) and 11 growth and differentiation factors (GDFs). All members of the TGFβ cytokine family are synthesised as large precursor polypeptides that require proteolytic processing by proprotein convertase (PC) enzymes either intracellularly or extracellularly. The family includes a number of antagonistic ligands: left-right determination factor 1 and 2 (LEFTY1 and LEFTY2) antagonise Nodal, inhibin antagonises activins and BMP3 antagonises other BMP members. The following members have alternative names indicated in parentheses: BMP15 (GDF9B), GDF2 (BMP9), GDF5 (BMP14), GDF6 (BMP13), MSTN (GDF8), GDF10 (BMP3B) and

GDF11 (BMP11). GDF15 is a distant member of the TGFβ family and signals through glial-derived neurotrophic factor receptor α-like (GFRAL). **(B)** Phylogenetic tree derived from the protein sequence alignment of the TGFβ family precursor polypeptides. Multiple sequence alignment was performed in Jalview (version 2.10.5) software (Waterhouse *et al.*, 2009) using the Clustal Omega sequence alignment programme. The phylogenetic tree was calculated using the neighbour-joining (NJ) method (Saitou and Nei, 1987). Abbreviations: AMH (Anti-Muellerian hormone), inhibin-α (INHA), inhibin-β (INHB), MSTN (Myostatin).

Ligands of the TGFβ family, along with those of the nerve growth factor (NGF) and platelet-derived growth factor (PDGF) families all share a similar, unique structural motif known as a cysteine knot (Sun and Davies, 1995). The presence of this cysteine knot motif occurs due to the formation of intramolecular disulphide bonds between pairs of highly conserved cysteine residues. In addition to these, a single intermolecular disulphide bond is formed between corresponding cysteine residues present within each monomer that enables dimer formation. Sequence alignment of the three different TGFβ isoforms (TGFβ1-3) encoded in the mammalian genome determines that they share over 70 percent sequence identity and reveals the presence of nine highly conserved cysteine residues in each polypeptide chain. Elucidation of the crystal structure of the TGFβ2 isoform revealed that eight of these invariant cysteine residues form four intramolecular disulphide bonds between Cys7 and Cys16, Cys15 and Cys78, Cys44 and Cys109 and between Cys48 and Cys111. The ninth cysteine residue, Cys77, forms an intermolecular disulphide bond with the corresponding cysteine residue of another TGFβ2 monomer, and along with hydrophobic interactions, is responsible for stabilisation of the active TGFβ2 homodimer (Daopin *et al.*, 1992; Schlunegger and Grütter, 1992). The majority of the other members in the TGFβ family possess seven conserved cysteine residues that correspond to those present in the TGFβ isoforms, however they lack the two amino-terminal residues and thus only form three intramolecular disulphide bonds.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TGFB1/289-390
TGFB2/313-414
TGFB3/311-412
NODAL/243-347
LEFTY1/259-359
LEFTY2/259-359
AMH/458-560
INHA/258-366
INHBA/317-426
INHBB/299-407
INHBC/243-352
INHBE/243-350
BMP2/292-396
BMP3/366-472
BMP4/304-408
BMP5/349-454
BMP6/408-513
BMP7/326-431
BMP8A/297-402
BMP8B/297-402
BMP10/319-424
BMP15/287-392
GDF1/263-372
GDF2/323-429
GDF3/260-364
GDF5/396-501
GDF6/350-455
GDF7/345-450
MSTN/277-375
GDF9/349-454
GDF10/372-478
GDF11/309-407
GDF15/207-308

Figure 1B. Sequence alignment of all 33 members of the TGFβ family of cytokines
All the members of the TGFβ family of cytokines share a distinctive structural motif referred to as a cysteine knot, which occurs due to the formation intramolecular disulphide bonds between pairs of highly conserved cysteine residues. In addition, a single intermolecular disulphide bond is also formed between corresponding cysteine residues present within each monomer that facilitates dimer formation. The three prototypical TGFβ isoforms contain nine invariant cysteine residues, eight of which participate in the formation of four intramolecular disulphide bonds and one which contributes to the intermolecular disulphide bond. The majority of the other TGFβ family members possess seven conserved cysteine residues that correspond to those present within the TGFβ isoforms and as a consequence only form three intramolecular disulphide bonds. Exceptions to this are the four inhibin-β monomers which contain the nine conserved cysteine residue arrangement as observed in the TGFβ isoforms and five family members (BMP15, GDF3, GDF9, LEFTY1 and LEFTY2) which only contain six conserved cysteine residues. As a result, it is uncertain whether these four members form dimers in order to be biologically active. The asterisks denote the seven conserved cysteine residues present within the prototypical TGFβ isoforms and the majority of other members (with exceptions detailed above). Multiple sequence alignment was performed in Jalview (version 2.10.5) software (Waterhouse *et al.*, 2009) using the Clustal Omega sequence alignment programme.

The fourth cysteine in each monomer participates in disulphide-linked dimerisation. Exceptions to this are the four inhibin-β monomers (A, B, C and E), which have the corresponding nine cysteine residue arrangement as observed in the TGFβ isoforms. Furthermore, some TGFβ family members (specifically BMP15, GDF3, GDF9, LEFTY1 and LEFTY2) only possess six conserved cysteine residues, compared with the usual seven-cysteine or nine-cysteine arrangement. In these instances, the members lack the fourth cysteine involved in ligand dimerisation and it is therefore uncertain as to whether they form disulphide-bonded dimers or utilise an alternate means to do. As such, the exact physiological functions of these six-cysteine TGFβ family members requires further investigation.

As previously alluded to, biologically active TGFβ family members exist as dimers and have been predominantly studied as homodimers. An exception to this is the inhibin subfamily, members of which form heterodimers consisting of an α subunit covalently associated with a β subunit via a disulphide bond. Inhibin A is a heterodimer of the inhibin α and inhibin β_A monomers, whereas Inhibin B is composed of inhibin α and inhibin β_B monomers. In contrast, the three activin isoforms, activin A, activin B and activin AB, exist as homodimers of inhibin β monomers (composed of βA/βA, βB/βB and βA/βB respectively)

(Namwanje and Brown, 2016). The binding of active TGFβ cytokines to cognate receptors present on the cell surface initiates the intracellular signalling pathway.

1.1.2 Signalling receptors of the TGFβ family of ligands

The TGFβ family of cytokines elicit their biological function by binding to a family of structurally related transmembrane receptors that are classified as either type I or type II receptors. Compared with other broad classes of transmembrane signalling receptors, such as the multipass G-protein-coupled receptors (GPCRs) that transduce signals from Wnt or Hedgehog ligands (Frizzled, Patched and Smoothened families respectively) or single pass transmembrane receptor tyrosine kinases (RTKs) that transduce signals from a diverse array of cytokines including epidermal growth factor (EGF), insulin and insulin-like growth factors (IGFs) and fibroblast growth factors (FGFs), the TGFβ type I and type II receptors are unique in that they possess serine-threonine protein kinase activity. This family of serine-threonine kinase receptors represents the only known signalling receptors for TGFβ family members and no other cytokines have been reported to signal through these receptors. Interestingly, both the type I and type II receptors have also been reported to exhibit tyrosine kinase activity. In the case of the type II receptor, it is able to autophosphorylate three tyrosine residues (Tyr259, Tyr336 and Tyr424) within its cytoplasmic domain, although the levels are considerably lower compared with autophosphorylation on serine and threonine residues. Mutation of these tyrosine residues to phenylalanine residues inhibited the kinase activity of the type II receptor *in vitro*, suggesting that autophosphorylation of tyrosine residues may have an autoregulatory function (Lawler *et al.*, 1997). In addition, the type I receptor has been shown to phosphorylate tyrosine residues on substrate proteins and this is important for non-canonical TGFβ signalling (discussed in further detail in section 1.2.5) (Lee *et al.*, 2007).

The human genome encodes 12 members of the serine-threonine kinase receptors, seven type I receptors and five type II receptors. The seven type I receptors are comprised of TGFβR1 (ALK5), ACVR1B (ALK4), ACVR1C (ALK7), ACVRL1 (ALK1), ACVR1A (ALK2), BMPR1A (ALK3) and BMPR1B (ALK6). The five type II receptors include TGFβR2, ACVR2, ACVR2B, BMPR2 and AMHR2 (Schmierer and Hill, 2007). This TGFβ family of signalling receptors represents a distinct family (termed STRK) within the tyrosine kinase-like (TKL) group of the human kinome (Manning *et al.*, 2002).

The type I and type II receptors are both glycoproteins that consist of approximately 500 amino acid residues, comprising a relatively short (~150 amino acid residues) amino-terminal extracellular domain, a single transmembrane domain and a carboxy-terminal intracellular serine-threonine kinase domain (Shi and Massagué, 2003). Although the type I and type II receptors are both serine-threonine kinases, they share only 30 to 40 percent sequence homology in their intracellular domains (Ebner *et al.*, 1993). In addition, the type I receptors possess unique features that are not present in type II receptors, notably a highly conserved 30 amino acid glycine-serine repeat sequence (SGSGSG; termed the GS domain) located immediately amino-terminal to the intracellular kinase domain (Wrana *et al.*, 1994; Massagué, 1998).

The TGFβ family of ligands initiate intracellular signalling pathways by inducing the formation of heterotetrameric complexes of transmembrane serine-threonine kinase receptors, however two different mechanisms of receptor activation have been identified depending on the ligand involved. TGFβ and activin ligands exhibit high affinity for the type II receptor but do not interact with isolated type I receptors. Therefore, ligand-binding to type II receptors occurs first, followed by interaction with the type I receptor to form a large heteromeric complex containing the ligand dimer and homodimers of the type II and type I receptors. By contrast, receptor activation induced by members of the BMP subfamily, such as BMP-2 and BMP-4, occurs via an alternate mechanism. In this case, the BMP ligands display a higher affinity for the extracellular ligand-binding domain of the type I receptor compared to that of the type II receptor. The assembled type I receptor-ligand dimer complex subsequently has a higher binding affinity for the type II receptor (Shi and Massagué, 2003). In both cases, the ligand functions to bring specific pairs of type I and type II receptors in close proximity. The type II receptor is believed to be a constitutively active kinase and when in proximity to the type I receptor it is able to phosphorylate multiple serine and threonine residues within the TTSGSGSG sequence of the intracellular GS domain (Wrana *et al.*, 1994). Phosphorylation of residues within the GS domain of the type I receptor is critical for its activation and ability to propagate signals from TGFβ family cytokines (Wieser, Wrana and Massagué, 1995). Furthermore, along with the residues within the GS domain, the type II receptor can also phosphorylate an additional serine residue (Ser165) located in the juxtamembrane region of the type I receptor. This phosphorylation event was demonstrated to modulate specific TGFβ-induced cellular responses in a positive or negative manner

(Souchelnytskyi *et al.*, 1996). In addition to phosphorylation, the activation of the type I receptor is also regulated by the binding of an inhibitory protein, the immunophilin FK506-binding protein 12 (FKBP12) (Wang, Donahoe and Zervos, 1994). In the absence of TGFβ ligands, FKBP12 binds to the unphosphorylated GS domain of the type I receptor and subsequently dissociates when ligand-induced, type II receptor-mediated phosphorylation of the type I receptor occurs (Wang *et al.*, 1996). The unphosphorylated GS domain adopts a helix-loop-helix structure in which the regulatory phosphorylation sites are located within the loop between the two helices (termed the GS loop). The crystal structure of the type I receptor in complex with FKBP12 revealed that the receptor adopts a kinase inactive conformation which is maintained by interactions between the unphosphorylated GS loop and the amino-terminal lobe (N-lobe) of the kinase domain. FKBP12 binds directly to two highly conserved leucine residues (Leu195 and Leu196) within one of the GS domain helices and maintains the GS loop in an ordered inhibitory conformation (Huse *et al.*, 1999). In the absence of FKBP12, the GS loop region is flexible and incapable of adopting this inhibitory conformation (Huse and Kuriyan, 2002). In addition to stabilising the inactive conformation of the type I receptor kinase domain, the binding of FKBP12 to the GS domain also obscures the type II receptor kinase phosphorylation sites. Therefore, it has been proposed that the function of FKBP12 is to impede the activation of heteromeric TGFβ receptor complexes that may occur in the absence of TGFβ ligands due to the innate affinity exhibited by type I and type II receptors towards one another, thereby preventing any spontaneous ligand-independent signalling (Chen, Liu and Massague, 1997).

Additional non-catalytic accessory receptors have been identified that regulate the interaction of certain TGFβ family ligands to cognate type I and type II receptors. A prominent example is the accessory receptor betaglycan (alternatively referred to as the TGFβ type III receptor), a membrane-anchored proteoglycan that mediates the binding of TGFβ isoforms to the type II receptor (López-Casillas *et al.*, 1991; Wang *et al.*, 1991). Betaglycan is composed of a large extracellular domain which is modified by multiple glycosaminoglycan chains followed by a transmembrane domain and short intracellular domain. It is structurally related to another TGFβ family accessory receptor endoglin, a glycoprotein that is expressed at high levels in endothelial cells and is able to bind to a number of heteromeric receptor complexes. However, unlike betaglycan, it was initially reported that endoglin is unable to bind the ligand on its own, requiring the presence of a ligand-binding type II receptor in order to do so.

Endoglin does not appear to alter the formation or ligand-binding affinity of the receptor complexes, nor does it affect the kinase activity of the receptor complex (Barbara, Wrana and Letarte, 1999). Recent research has determined the crystal structure of the extracellular domain of endoglin in complex with the BMP-9 homodimer and proposed that endoglin may function to secure a pool of BMP-9 ligands at the plasma membrane where it can concomitantly bind the type I receptor ALK1 and subsequently interact with a type II receptor (Saito *et al.*, 2017). However, further research is required to investigate the mechanisms by which endoglin interacts with other TGFβ ligands and receptors and thus determine its precise function in TGFβ signalling. Although betaglycan is able to bind all TGFβ isoforms, it appears to be most important for TGFβ2, particularly during embryonic development (Brown *et al.*, 1999; Stenvers *et al.*, 2003). It has been postulated that the cell-type-specific expression of accessory receptors may determine whether a cell can or cannot respond to specific TGFβ ligands that require accessory receptors in order to activate their cognate heterotetrameric receptor complex (Schmierer and Hill, 2007).

Once activated by type II receptor-mediated phosphorylation, the type I receptors are able to propagate the signals of TGFβ ligands via the phosphorylation of downstream substrates.

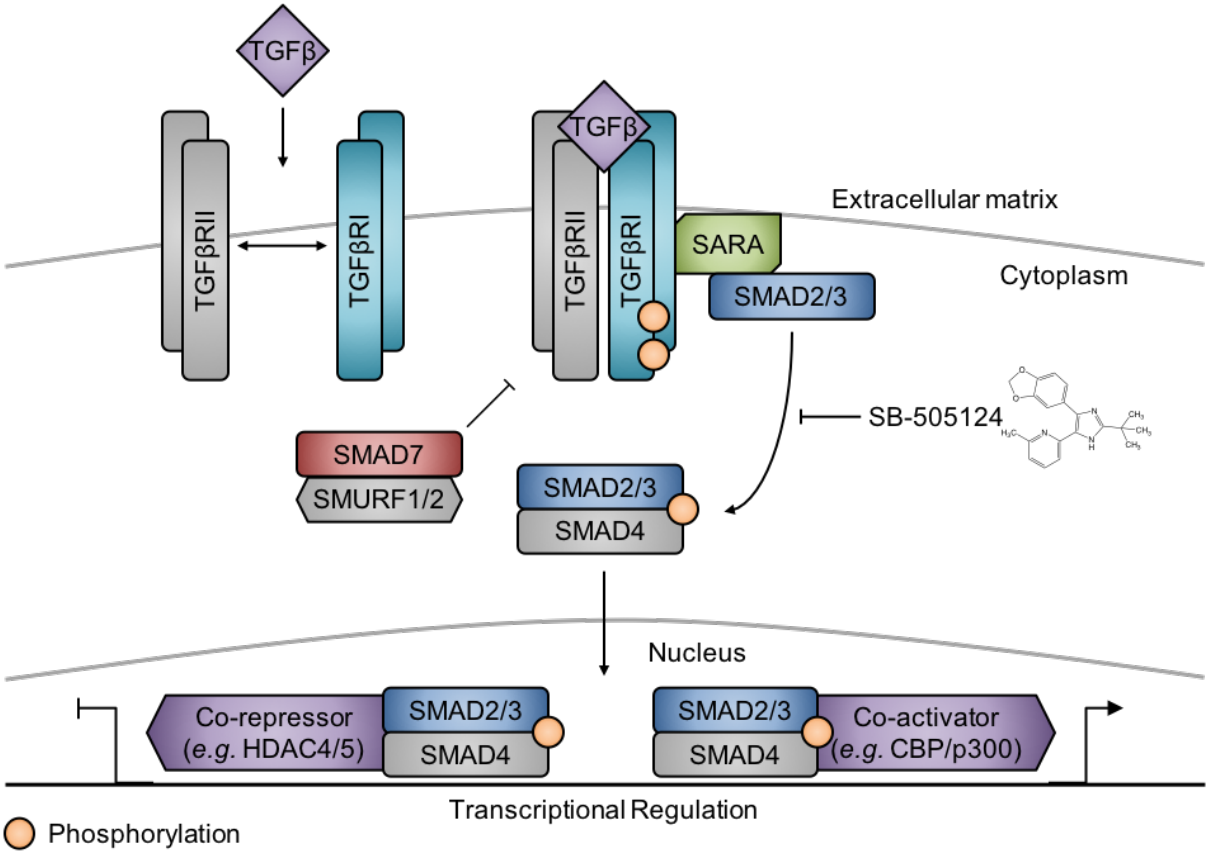


Figure 1C. Overview of the core components of SMAD-dependent TGFβ signalling
The binding of TGFβ ligands to cognate cell surface receptor serine-threonine kinases initiates the signalling pathway by inducing the formation of heterotetrameric receptor complexes consisting of type I receptor dimers and type II receptor dimers. In close proximity, the type II receptor is able to phosphorylate and hence activate the type I receptor. The activated type I receptor kinase subsequently phosphorylates two serine residues within the conserved carboxy-terminus of the R-SMAD proteins SMAD2 and SMAD3. Phosphorylated R-SMADs form heteromeric complexes with the mediator SMAD4 and translocate into the nucleus, where they co-operate with additional transcription factors or chromatin modifying enzymes to regulate target gene transcription. TGFβ induces the transcription of inhibitory SMAD proteins (SMAD6/SMAD7) which participate in a feedback mechanism by competing with R-SMADs for receptor binding or directing E3 ubiquitin ligases and/or DUBs to the activated receptor complex.

1.1.3 SMAD proteins – the mediators of the canonical TGFβ signalling pathway

The SMAD proteins are a family of structurally related transcription factors which function as the predominant intracellular mediators of the TGFβ signalling pathway (*N.B.* the SMAD-dependent pathway is referred to as the canonical TGFβ signalling pathway). The nomenclature SMAD is a portmanteau derived from the fact that they are the vertebrate

homologs of mothers against decapentaplegic (MAD) and Sma (referring to smaller than wild type phenotype) identified as the intracellular signal transducers of TGFβ family cytokines in *Drosophila melanogaster* (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995) and *Caenorhabditis elegans* (Savage *et al.*, 1996) respectively. The mammalian genome encodes eight SMAD proteins, designated SMAD1 to SMAD8 (which is also referred to as SMAD9), which can be classified into three subfamilies based on a number of structural and functional considerations. Five SMAD proteins (SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8) are substrates for the type I serine-threonine kinase receptors and are thus collectively referred to as receptor-regulated SMADs (R-SMADs). SMAD4 functions as a common mediator of the R-SMADs and is therefore known as a co-SMAD. Both SMAD6 and SMAD7 function as inhibitory SMAD proteins (I-SMADs) and can antagonise the signalling pathway in a number of different ways, including binding to the type I receptor, recruiting E3 ubiquitin-protein ligases to the receptor complex or by interfering in the formation of the R-SMAD-SMAD4 complex (Hata and Chen, 2016).

Amongst the R-SMAD proteins, SMAD2 and SMAD3 are primarily phosphorylated by the type I receptors for the TGFβ subfamily (TGFβR1, ACVR1B and ACVR1C), whereas SMAD1, SMAD5 and SMAD8 are mainly phosphorylated by type I receptors for BMP subfamily ligands (ACVRL1, ACVR1A, BMPR1A and BMPR1B) (David and Massagué, 2018). The division of the pathway into TGFβ members signalling via SMAD2 and SMAD3, and BMP members signalling via SMAD1, SMAD5 and SMAD8 is relatively useful distinction, however more recent research has revealed a greater complexity to the system. For example, in a number of different mammalian epithelial cell lines, it has been demonstrated that TGFβ1 can induce the phosphorylation of SMAD1/SMAD5 in addition to SMAD2/SMAD3 and that this may result in the formation of mixed R-SMAD complexes. It was proposed that this is mediated by the formation of novel heteromeric receptor complexes containing homodimeric type II receptors associated with heterodimeric type I receptors (Daly, Randall and Hill, 2008). Recent research from the same group aimed at further elucidating the mechanism and functional relevance of this non-canonical form of TGFβ-induced SMAD1/SMAD5 phosphorylation suggested that the kinetics of TGFβ-induced SMAD1/SMAD5 phosphorylation appears to be different to that of SMAD2/SMAD3 phosphorylation, requiring the serine-threonine kinase activity of two different type I receptors, TGFβR1 and ACVR1 (ALK5 and ALK2 respectively). Interestingly, it was observed that TGFβ-activated TGFβR1

could phosphorylate and activate ACVR1, which subsequently phosphorylated SMAD1. Furthermore, the phosphorylated SMAD1/SMAD5 complexes formed in response to TGFβ stimulation appear to be transcriptionally competent and required for TGFβ-induced epithelial-mesenchymal transition (EMT) (Ramachandran *et al.*, 2018). This presents a new paradigm for TGFβ receptor activation and enhances the complexity of the signalling pathway. Whether these observations are restricted to specific cell types or contexts or are more pervasive is an area of ongoing research.

The R-SMADs are comprised of approximately 500 amino acid residues and consist of highly conserved globular amino-terminal MAD homology 1 (MH1) and carboxy-terminal MAD homology 2 (MH2) domains connected via a less conserved linker region. At the amino-terminus, within the MH1 domain, are putative nuclear localisation signal (NLS) motifs consisting of lysine-rich sequences. At their extreme carboxy-terminus the R-SMADs contain a conserved Ser-Ser-Xxx-Ser (SSXS) motif that is phosphorylated on the last two of the serine residues by the activated type I receptor kinase (Hata and Chen, 2016). The co-SMAD SMAD4 is similar to the R-SMADs, containing the conserved MH1 and MH2 domains, however it does not possess the SSXS motif and thus cannot be phosphorylated by the type I receptor kinases. The MH1 domain of the R-SMADs and SMAD4 facilitates DNA binding and the crystal structures of SMAD1, SMAD3 and SMAD4 MH1 domains reveal that it consists of four α-helices and three sets of antiparallel β-hairpin strands (β1-β4, β2-β3 and β5-β6) (Macias, Martin-Malpartida and Massagué, 2015). The interaction of the MH1 domain with DNA is mediated by a conserved 11-amino acid residue β-hairpin loop structure formed by the β2 and β3 strands that interacts with the major groove of DNA in a base-specific manner (Shi *et al.*, 1998). An exception to this is SMAD2, the most abundant isoform of which contains an insertion (encoded by exon 3) that perturbs DNA binding (Massagué, Seoane and Wotton, 2005). Interestingly, an alternative splice variant of SMAD2 in which exon 3 has been deleted (SMAD2Δexon3) exhibits DNA-binding and transcriptional activity similar to that of SMAD3 (Yagi *et al.*, 1999). Early research into the sequence specificity of SMAD MH1 domains identified the eight base-pair palindromic sequence 5'-GTCTAGAC-3' (termed the CAGA motif) as the preferred binding sequence of SMAD3 and SMAD4, albeit with relatively low affinity (Zawel *et al.*, 1998). Furthermore, R-SMADs that transduce signals from BMP family ligands, namely SMAD1 and SMAD5, were shown to preferentially bind to GC-rich sequences within the promoter regions of BMP target genes (Kusanagi *et al.*, 2000; Morikawa *et al.*,

2011). Therefore, these collective observations resulted in the paradigm that the CAGA motif functions as a SMAD binding element (SBE) for TGFβ subfamily ligands, whereas GC-rich sequences present within target gene promoter regions function as BMP responsive elements (BRE) for BMP subfamily ligands. However, given the identical sequence of the DNA-binding β-hairpin loop present in all the R-SMADs (Shi *et al.*, 1998; Macias, Martin-Malpartida and Massagué, 2015) and subsequent observations that SMAD3 and SMAD4 can bind to GC-rich sequences that do not contain the CAGA motif, this dichotomy of R-SMAD-DNA binding is somewhat misleading. Indeed, recent structural analysis demonstrated that TGFβ-activated SMAD3, BMP-activated SMAD1/SMAD5 and SMAD4 all recognise the common consensus sequence GGC(G/C)(C/G) (Martin-Malpartida *et al.*, 2017). Clusters of these five base-pair sequences, termed the 5GC SBE motif, are highly represented in the promoter and enhancer regions of both TGFβ and BMP target genes. Therefore, it is proposed that both TGFβ-activated SMAD2/SMAD3 and BMP-activated SMAD1/SMAD5 both recognise common DNA sequences, and it is the differential binding of SMAD2/SMAD3 and SMAD1/SMAD5 to different transcription co-factors that determines target gene specificity (David and Massagué, 2018).

The MH2 domain is a highly conserved globular domain present within all SMAD family members and is responsible for mediating protein-protein interactions. The crystal structure of the SMAD2 MH2 domain in complex with the SMAD-binding domain (SBD) of SMAD anchor for receptor activation (SARA) revealed that it consists of a central β-sheet structure with an amino-terminal loop-helix region and carboxy-terminal three-helix bundle (Wu *et al.*, 2000). In the context of R-SMADs, the MH2 domain is responsible for interaction with the type I receptors. A pocket of basic residues within the R-SMAD MH2 domain interacts with phosphorylated serine residues within the GS domain of the activated type I receptor kinase (Massagué, Seoane and Wotton, 2005). The specificities of this interaction are determined by the L45 loop sequences in the receptor and the loop-helix sequence in the R-SMAD MH2 domain. A series of contiguous hydrophobic regions (termed the hydrophobic corridor) on the surface of the R-SMAD MH2 domain mediate interaction with cytoplasmic retention proteins, such as SARA, and DNA-binding transcriptional co-regulators (discussed in further detail in the subsequent section).

As previously mentioned, the conserved MH1 and MH2 domains of SMAD proteins are connected via a divergent linker. This linker region contains a proline-tyrosine (PPXY)

motif which binds to specific E3 ubiquitin-protein ligases as well as multiple regulatory phosphorylation sites. Thus, the linker region is critical for the regulation of SMADs and integration of crosstalk from other intracellular signalling pathways (discussed in further detail in section 1.3.4).

The inhibitory SMADs (SMAD6 and SMAD7) possess the conserved carboxy-terminal MH2 domain however they do not contain the MH1 domain found in R-SMADs and SMAD4. In addition, they lack the carboxy-terminal SSXS motif and are therefore not subject to type I receptor-mediated phosphorylation. The expression of I-SMADs can be induced by both TGFβ (Nagarajan *et al.*, 1999; Denissova *et al.*, 2000) and BMP family ligands (Ishida *et al.*, 2000; Benchabane and Wrana, 2003) to provide negative feedback mechanisms. Negative regulation of TGFβ family signalling by I-SMADs occurs through a number of different mechanisms and at various stages of the signalling pathway. For example, SMAD7 can directly bind to the activated type I receptor kinases, thereby interfering with the interaction between the activated receptor complex and R-SMADs and preventing their phosphorylation. Four basic residues (Lys312, Lys316, Lys401 and Arg409) within the MH2 domain of human SMAD7 appear to be crucial for interaction with the L45 loop of the activated type I receptor (Mochizuki *et al.*, 2004). Furthermore, SMAD6 can compete with SMAD4 for binding to receptor-activated SMAD1 and thereby inhibit BMP signalling through the formation of an inactive heterotrimeric SMAD1-SMAD6 complex (Hata *et al.*, 1998). Moreover, research has also demonstrated that the homologous to E6-AP carboxyl terminus (HECT) E3 ubiquitin-protein ligase SMURF1 can both directly and indirectly, via interaction with SMAD6, associate with SMAD1/SMAD5 to induce their ubiquitylation and subsequent proteasomal degradation (Zhu *et al.*, 1999; Murakami *et al.*, 2003). SMAD7 has been shown to interact with both SMURF1 (Ebisawa *et al.*, 2001) and SMURF2 (Kavsak *et al.*, 2000) to induce the ubiquitylation and degradation of activated receptor complexes via proteasomal and lysosomal pathways. In addition to the SMURF E3 ubiquitin-protein ligases, SMAD7 has also been shown to interact with growth arrest and DNA damage protein 34 (GADD34), a regulatory subunit of the protein phosphatase 1 (PP1) holoenzyme, to promote the dephosphorylation of the activated TGFβR1 receptor kinase (Shi *et al.*, 2004). GADD34 functions to recruit the catalytic subunit of PP1 (termed PP1c) and recruitment of the GADD34-PP1c complex to activated TGFβR1 receptor by SMAD7 induces its dephosphorylation and hence inactivation.

Therefore, SMAD6 and SMAD7 function to antagonise the TGFβ signalling pathway through different mechanisms including competition with R-SMADs for receptor interaction, proteasomal degradation of activated receptor complexes and R-SMADs by SMURFs and dephosphorylation of activated type I receptors by PP1. The regulation of TGFβ signalling by reversible protein phosphorylation and ubiquitylation will be discussed in greater detail in the subsequent sections 1.3.4 and 1.3.3 respectively.

Once phosphorylated at their carboxy-terminal SSXS motif by the type I receptor kinases, R-SMADs interact with their common binding partner SMAD4 to form heterotrimeric complexes, comprising two R-SMADs and SMAD4. The R-SMAD-SMAD4 complexes subsequently accumulate in the nucleus, where they bind to regulatory regions of target genes to either activate or repress gene transcription.

1.1.4 Transcriptional control by SMAD proteins

Signalling by TGFβ family cytokines can elicit a diverse array of cellular responses by regulating programmes of gene expression. However, the precise molecular basis of how a conceptually simple signal transduction pathway can regulate a broad range of responses depending on cell type or context has remained an open question. Research over the previous two decades has provided new insights into how SMAD proteins cooperate with sequence-specific DNA-binding transcription factors or modifiers of chromatin structure in a highly contextual manner. These insights have provided a unified model of how signalling through TGFβ ligands and SMAD transcription factors initiates diverse and specific responses depending on the biological context.

The DNA-binding affinity of SMADs is relatively weak (Shi *et al.*, 1998; Chai *et al.*, 2003) and the binding of SMADs to short DNA sequences (5GC SBE motifs) is not particularly specific, therefore the interaction of SMADs with additional transcription factors is proposed as the principal determinant of high-affinity and high-specificity recruitment of SMADs to DNA (Hill, 2016). However, the function of these SMAD-cooperating transcription factors may occur through different mechanisms. For example, they may be fundamentally required to recruit activated SMAD complexes to DNA, as demonstrated by the transcription factor forkhead box protein H1 (FOXH1). FOXH1, previously known as forkhead activin signal transducer 1 (FAST1),

was the first SMAD-interacting transcription factor to be identified and is required for the recruitment of SMAD2-SMAD4 complexes in response to activin stimulation in *Xenopus laevis* embryos (Chen, Rubock and Whitman, 1996; Chen *et al.*, 1997). FOXH1 mediates the recruitment of an activated SMAD2-SMAD4 complex to the activin-responsive element (ARE) within the promoter region of Mix.2, an immediate-early response gene, during early *Xenopus* embryogenesis. In this way, FOXH1 functions as an archetypal lineage-determining transcription factor (LDTF) for SMADs, which are signal-driven transcription factors (SDTFs). LDTFs are defined as cell type-specific transcription factors which dictate the binding of SDTFs at specific genomic loci in order to establish gene expression patterns that define cell identity and function (David and Massagué, 2018).

Activated SMAD complexes can regulate the transcription of other transcriptional regulators which in turn bind to the SMAD complex to regulate the transcription of other target genes (Hill, 2016). An example of this self-enabling transcriptional response occurs with the transcriptional repressor activating transcription factor 3 (ATF3). In the context of epithelial cells, TGFβ-activated SMAD3 can directly induce the expression of ATF3, which subsequently forms a complex with SMAD3 that directly represses the transcription of DNA-binding protein inhibitor ID-1 (Kang, Chen and Massagué, 2003). Downregulation of ID-1 contributes to the cytostatic effect observed in certain epithelial cell lines in response to TGFβ.

In addition to DNA-binding transcription factors, SMADs can also interact with various coactivators and corepressors that regulate the transcriptional response by modifying chromatin structure. Transcriptionally permissive promoter regions are associated with high levels of acetylation on conserved lysine residues of histone proteins. Activated R-SMAD-SMAD4 complexes have been shown to interact with the histone acetyltransferases (HATs) E1A-associated protein p300 (EP300; previously known as p300) (Feng *et al.*, 1998) and GCN5 (Kahata *et al.*, 2004) and these interactions are required for TGFβ-induced gene transcription. Further evidence for the role of chromatin modification in SMAD-mediated gene transcription was obtained from an *in vitro* transcription system using recombinant phosphorylated SMAD2-SMAD4 complexes. The phosphorylated SMAD2-SMAD4 complex was unable to induce transcription from naked DNA templates, however they could efficiently activate transcription from chromatin templates, suggesting that transcriptional regulation mediated by activated SMAD complexes involves, at least in part, modification of chromatin structure

(Ross *et al.*, 2006). SMAD complexes can also mediate transcriptional repression by removing histone acetylation modifications at promoter regions of target genes via the enzymatic action of histone deacetylases (HDACs). Research has demonstrated that TGFβ-activated SMAD3 can directly associate with the class IIa HDACs 4 and 5 to deacetylate histone H4 at the promoter for osteocalcin, a protein required for osteoblast differentiation (Kang *et al.*, 2005). Osteocalcin is a target gene for the osteoblast-specific transcription factor runt-related transcription factor 2 (RUNX2) (also known as CBFA1). Previous research demonstrated that SMAD3 interacts directly with RUNX2 and represses its transcriptional activity (Alliston *et al.*, 2001). Therefore, SMAD3-mediated recruitment of HDAC4 and HDAC5 to SMAD3-RUNX2 complexes inhibits the transcriptional function of RUNX2 and consequently represses the transcription of RUNX2 target genes during osteoblast differentiation.

1.1.5 SMAD-independent signalling by TGFβ family members

As previously alluded to, the interaction of the TGFβ family of cytokines with cognate transmembrane serine-threonine kinase receptors and subsequent activation of intracellular SMAD transcription factors constitutes the canonical TGFβ signal transduction pathway. However, in addition to SMAD-mediated signalling, it has become increasingly appreciated that signalling through SMAD-independent pathways represents an important contribution to cellular responses elicited by TGFβ family cytokines. Research has indicated that activated TGFβ receptor complexes can signal through other signal transducers including mitogen-activated protein kinase (MAPK) pathways, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and Rho family GTPases (Zhang, 2017). These are collectively referred to as *non-canonical* TGFβ signalling pathways and may function alone or in conjunction with canonical SMAD signalling to mediate the cellular response to TGFβ ligands.

Initial evidence for the role of MAPK pathways in TGFβ signalling came from the observation that TGFβ stimulation of epithelial cells resulted in the rapid activation of the small GTPase Ras (Mulder and Morris, 1992). Further research demonstrated that TGFβ is capable of activating three different MAPK pathways: extracellular signal-regulated kinase (ERK) MAPK, c-Jun N-terminal kinase (JNK) MAPK and p38 MAPK (Derynck and Zhang, 2003). TGFβ stimulation can result in the sequential activation of the serine-threonine protein

kinases RAF (a MAPKKK), MEK1/2 and ERK1/2, however this occurs at lower levels compared to cytokine stimulation through receptor tyrosine kinases (Mulder, 2000). Upon TGFβ stimulation, the activated type I receptor directly interacts and phosphorylates the adaptor protein ShcA on serine and tyrosine residues (Lee *et al.*, 2007). Phosphorylation of ShcA induces its association with another adaptor protein, growth factor receptor-bound protein 2 (GRB2), and the guanine nucleotide exchange factor (GEF), son of Sevenless (SOS). The ShcA-GRB2-SOS complex converts Ras into its active guanosine-5'-triphosphate (GTP)-bound conformation which ultimately results in the activation of ERK1 and ERK2 (Dhillon *et al.*, 2007).

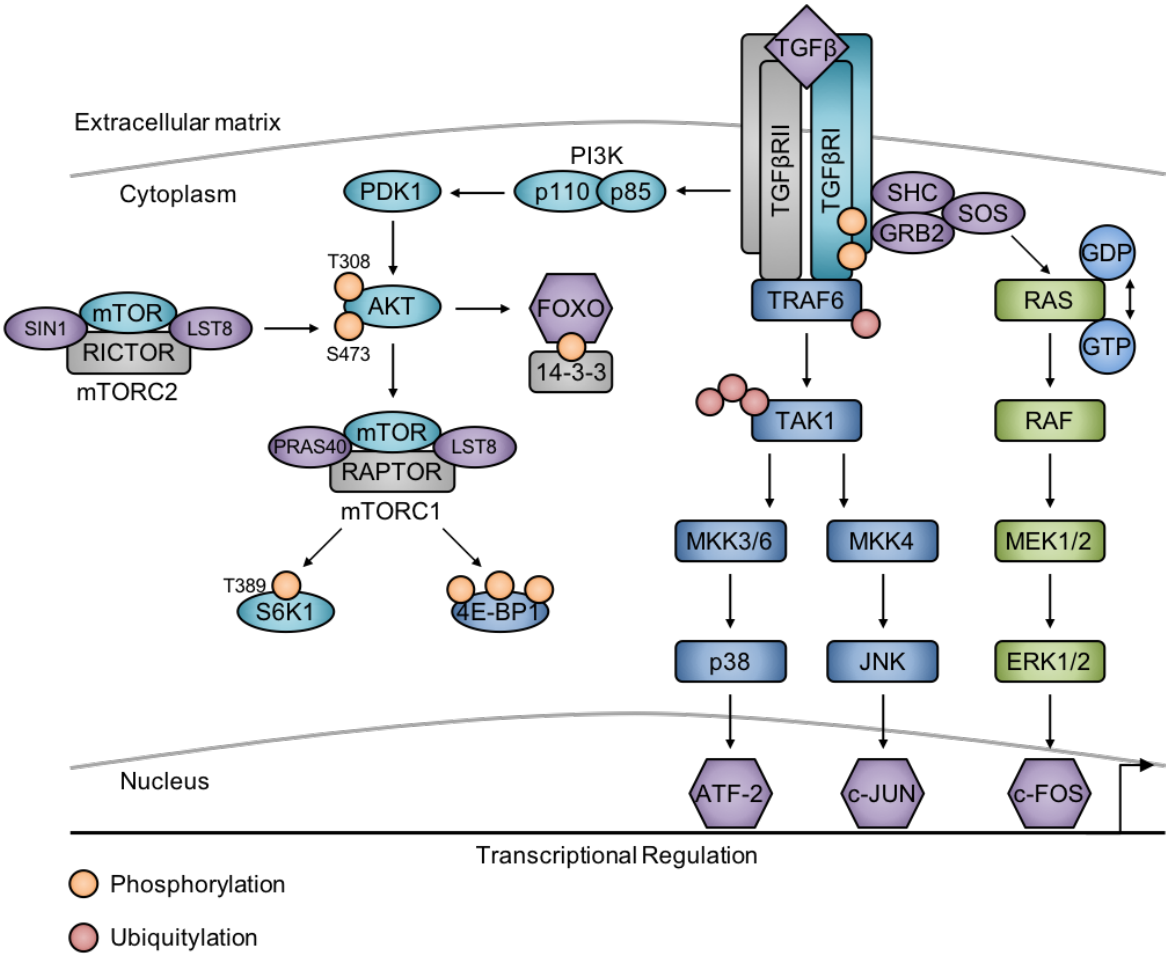


Figure 1D. Overview of SMAD-independent TGFβ signalling pathways
In addition to the activation of SMAD transcription factors (referred to as the canonical TGFβ pathway), the binding of TGFβ family cytokines to their cognate cell surface receptor kinases can also induce the activation of a number of additional signal transduction pathways

(collectively referred to as non-canonical TGFβ pathways). These non-canonical TGFβ signalling pathways primarily include the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. The non-canonical TGFβ signalling pathways may operate independently or in conjunction with canonical SMAD-dependent TGFβ signalling to mediate the cellular responses to TGFβ cytokines.

TGFβ can induce the rapid and transient activation of JNK and p38 MAPKs through the activation of the MAP kinase kinases (MKKs) MKK4 and MKK3/MKK6 respectively (Zhang, 2017). MKKs require activation by upstream protein kinases termed MAP kinase kinase kinases (MKKKs). TGFβ-activated kinase (TAK1) was identified as an MKKK that becomes activated in response to TGFβ stimulation and capable of mediating the activation of p38 MAPK (Yamaguchi *et al.*, 1995). Moreover, TAK1 was also shown to be required for activation of JNK MAPK in response to TGFβ. Stimulation of wild type mouse embryonic fibroblasts (MEFs) with TGFβ resulted in rapid phosphorylation of JNK, however JNK phosphorylation was abrogated in TAK1-deficient MEFs (Shim *et al.*, 2005). Activation of TAK1 can also be induced by signalling through the interleukin-1 (IL-1R) and Toll-like receptors (TLRs) (Wu and Arron, 2003) and requires the formation of Lys63-linked polyubiquitin (K63-Ub) chains, mediated in part by the E3 ubiquitin-protein ligase tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Cohen and Strickson, 2017). Similarly, activation of TAK1 by TGFβ requires TRAF6, which interacts with a consensus binding motif present within the TGFβ type I receptor. Interaction of TRAF6 with the type I receptor results in autoubiquitylation of TRAF6 and subsequent K63-linked polyubiquitylation of TAK1 on Lys34, resulting in its activation and subsequent activation of downstream JNK and p38 MAPKs (Sorrentino *et al.*, 2008; Yamashita *et al.*, 2008). However, the exact requirement for TAK1 in mediating TGFβ-induced phosphorylation of p38 MAPK remains somewhat uncertain. Recent research observed that TGFβ-induced phosphorylation of p38 MAPK still occurred in TAK1-deficient MEFs. By contrast, phosphorylation of p38 MAPK induced by IL-1 was completely abolished in the absence of TAK1 and restored upon expression of wild-type but not a catalytically inactive form of TAK1 (Sapkota, 2013). As with SMAD-dependent signalling, the nature of SMAD-independent pathways may be contingent on cell type or context.

In certain cell contexts, evidence suggests that TGFβ-induced activation of MAPK pathways occurs independently of SMAD activation (Yu, Hébert and Zhang, 2002; Itoh *et al.*,

2003). For example, the kinase activity of the TGFβ type I receptor is dispensable for the ability of TRAF6 to activate TAK1, whereas the interaction with the type I receptor and E3 ubiquitin ligase activity of TRAF are both required for TAK1 activation. Furthermore, SMAD-independent activation of MAPK pathways was demonstrated using a mutated form of the TGFβ type I receptor that is unable to activate SMAD2 or SMAD3 but retains its kinase activity. The L45 loop is situated within the kinase domain of the type I receptors and facilitates binding to R-SMADs, however mutation of three residues (Asn265, Asp267 and Asn268) abolished the SMAD-binding ability of the receptor. Despite this, the mutated type I receptor kinase was sufficient to activate p38 MAPK and induce phosphorylation of its substrate, cyclic AMP-dependent transcription factor ATF-2. A constitutively active form of the L45 loop mutant type I receptor was able to induce apoptosis in murine mammary epithelial cells however it was not sufficient to induce EMT (Yu, Hébert and Zhang, 2002). Furthermore, TGFβ-induced activation of p38 MAPK was also shown to promote apoptosis in a prostate cancer cell line (Edlund *et al.*, 2003). This suggests that TGFβ-induced p38 MAPK activation is sufficient for TGFβ-mediated apoptosis, however induction of EMT requires contribution from the SMAD-dependent pathway.

In addition to activation of MAPK pathways, research has also indicated that TGFβ family cytokines may also signal through class I PI3K and its downstream effector, the serine-threonine protein kinase AKT (otherwise known as protein kinase B; PKB) (Zhang, 2009). TGFβ1 stimulation can induce the phosphorylation of AKT at serine 473, which is prevented upon treatment with a PI3K inhibitor (Bakin *et al.*, 2000) and occurs independent of SMAD activation (Wilkes *et al.*, 2005). Similar to SMAD-dependent and MAPK pathways, TGFβ stimulation of the PI3K pathway appears to be cell-context dependent, occurring in some epithelial cell lines but not others. PI3K was found to be constitutively associated with the TGFβ type II receptor via its regulatory subunit p85, whereas association with the TGFβ type I was transient, requiring TGFβ stimulation (Yi, Shin and Arteaga, 2005). However, although the association between PI3K and the TGFβ receptors does not occur directly, it does require the kinase activity of the receptors. Expression of a kinase inactive, dominant negative form of the type II receptor inhibited the ligand-induced association between the p85 subunit and type I receptor and concomitant TGFβ stimulation of PI3K kinase activity. Moreover, overexpression of a constitutively active form of the type I receptor enhanced PI3K kinase activity, which was subsequently blocked by the use of a type I receptor kinase inhibitor (Yi,

Shin and Arteaga, 2005). Activation of the PI3K-AKT pathway by TGFβ results in activation of mammalian target of rapamycin (mTOR) during TGFβ-induced EMT. Activation of mTOR complex 1 (mTORC1) by TGFβ results in the phosphorylation of the downstream mTORC1 effectors ribosomal protein S6 kinase beta-1 (S6K1, also knowns as p70-S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which are direct regulators of translation initiation. The translational regulation resulting from TGFβ-mediated activation of mTORC1 and phosphorylation of S6K1 and 4E-BP1 enhanced cell size and protein content, and contributed to cell invasion, during TGFβ-induced EMT (Lamouille and Derynck, 2007). Subsequent research has also demonstrated that, in addition to activation of mTORC1, TGFβ can also induce the activation of mTOR complex 2 (mTORC2) during EMT. RNA interference (RNAi)-mediated depletion of rapamycin-insensitive companion of mTOR (RICTOR), a subunit of mTORC2 (Sarbassov *et al.*, 2004), prevented mTORC2 activation by TGFβ and also suppressed the expression of the transcription factor Snail (also termed zinc finger protein SNAI1). Snail expression is upregulated in response to TGFβ and functions to repress the transcription of E-cadherin, a fundamental event during EMT. Depletion of RICTOR attenuated the TGFβ-induced expression of Snail and resulted in the incomplete downregulation of E-cadherin, thereby contributing to the inhibition of TGFβ-induced EMT observed in RICTOR-depleted cells (Lamouille *et al.*, 2012). Therefore, although the activity of mTORC2 is not required for maintenance of an epithelial cell phenotype, TGFβ-induced mTORC2 activation is required for epithelial cells to transition to a mesenchymal, invasive phenotype.

PI3K-AKT signalling can also impact TGFβ-induced gene transcription by phosphorylating SMAD-interacting partners. For example, AKT can phosphorylate the transcription factor forkhead box protein O3 (FOXO3, previously known as FKHRL1), resulting in its association with 14-3-3 proteins and retention in the cytoplasm, thereby inhibiting the ability of FOXO3 to induce transcription of target genes (Brunet *et al.*, 1999). In response to TGFβ stimulation, FOXO transcription factors cooperate with SMAD3 to mediate the transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor $p21^{CIP1}$, thereby contributing to the TGFβ-mediated cytostatic response. Treatment of cells with a PI3K inhibitor enhanced the nuclear localisation of endogenous FOXO3 and augmented the induction of $p21^{CIP1}$ in response to submaximal concentrations of TGFβ (Seoane *et al.*, 2004).

Therefore, by regulating the subcellular location of FOXO transcription factors, PI3K-AKT signalling can negatively regulate SMAD-dependent gene transcription.

Collectively, these observations suggest complex regulatory interplay between TGFβ-induced SMAD-dependent and SMAD-independent pathways, which are important for cellular responses mediated by TGFβ cytokines such as apoptosis, EMT and cytostasis.

1.2 THE REGULATION OF TGFβ SIGNALLING

1.2.1 Regulation of TGFβ signalling by reversible post-translational modification (PTM)

TGFβ cytokines control many different cellular responses in a context-dependent manner and therefore complex mechanisms have developed to regulate the pathway and thus modulate the signalling outcome. One of the most prevalent mechanisms by which intracellular signalling pathways are regulated is through reversible post-translational modification (PTM) of proteins. It is therefore not surprising that a plethora of research has uncovered many ways in which reversible PTM can regulate components of the TGFβ signalling pathway, in particular protein phosphorylation and ubiquitylation. For the purpose of this section, I will focus the discussion on the reversible protein phosphorylation and ubiquitylation of TGFβ receptors and SMAD proteins.

1.2.2 Regulation of TGFβ receptors by reversible protein phosphorylation

As introduced in section 1.2.2, the type II TGFβ receptor is constitutively active transmembrane serine-threonine protein kinase that upon binding of a cognate extracellular TGFβ ligand, forms a heterotetrameric complex with the type I TGFβ receptor. The formation of this receptor complex enables the type II receptor to phosphorylate multiple serine and threonine residues within the cytoplasmic GS domain of the type I receptor (Wrana *et al.*, 1994; Wieser, Wrana and Massagué, 1995). This phosphorylation relieves inhibition mediated by the inhibitory protein FKBP12 (Chen, Liu and Massague, 1997; Huse *et al.*, 2001) and facilitates interaction between the type I receptor and the MH2 domain of R-SMAD proteins, allowing the activated type I receptor to phosphorylate two serine residues within the carboxy-terminal SSXS motif of the R-SMADs. The phosphorylation of the type I receptor can be reversed by the serine/threonine-protein phosphatase PP1 (Shi *et al.*, 2004). The regulatory subunit of PP1, termed GADD34, interacts with SMAD7, which facilitates recruitment to the activated type I receptor and promotes dephosphorylation of the receptor by the PP1 catalytic subunit (referred to as PP1c) (previously discussed in section 1.2.3). SMAD7 is upregulated in response to TGFβ (Nakao *et al.*, 1997) and thus its ability to facilitate receptor dephosphorylation represents one of its inhibitory functions and constitutes an

important negative feedback mechanism to modulate TGFβ signalling. Furthermore, in endothelial cells, it has been reported that TGFβ signalling through the type I receptor ACVRL1 (ALK1) can transcriptionally induce the PP1α isoform, which is subsequently recruited to ACVRL1 via interaction with SMAD7 (Valdimarsdottir *et al.*, 2006). SMAD7 can function to inhibit ACVRL1-mediated SMAD1/SMAD5 phosphorylation and therefore one of the proposed mechanisms by which this can occur is through PP1α-mediated dephosphorylation of the receptor.

1.2.3 Regulation of TGFβ receptors by reversible protein ubiquitylation

In addition to dephosphorylation, ubiquitin-mediated degradation of activated receptor complexes is another fundamental mechanism by which cells ensure a balanced response to TGFβ signals (Izzi and Attisano, 2004; Al-Salihi, Herhaus and Sapkota, 2012). Protein ubiquitylation involves the covalent conjugation of the 76-amino acid polypeptide ubiquitin to lysine residues of substrates via the sequential action of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-ligating enzymes in an ATP-dependent process (Hershko and Ciechanover, 1998; Kerscher, Felberbaum and Hochstrasser, 2006). This enzymatic cascade results in the formation of an isopeptide bond between the carboxy-terminal glycine residue of ubiquitin (G76) and the ε-amino group of a target lysine residue or the extreme amino terminus of a polypeptide. Furthermore, an isopeptide bond can also be formed between the ubiquitin G76 residue and the ε-amino group of a lysine residue within another ubiquitin molecule, thereby enabling the formation of polyubiquitin chains on substrate proteins. Ubiquitin contains seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) allowing the generation of seven possible homotypic chain types or numerous heterotypic (*i.e.* branched or mixed) linkage chain types (Dikic, Wakatsuki and Walters, 2009). Additionally, ubiquitin molecules can also be linked between the carboxy-terminal G76 residue and the amino-terminal methionine residue resulting in the formation of linear (M1) polyubiquitin chains. Therefore, ubiquitin modification of protein substrates may involve the conjugation of a single ubiquitin moiety (*i.e.* monoubiquitylation) or the formation of homotypic or heterotypic polyubiquitin chains resulting in a highly complex post-translational regulatory system.

The process of protein ubiquitylation can be reversed by the enzymatic action of deubiquitylating (DUB) enzymes, a large family of proteases which remove ubiquitin from substrate proteins by catalysing the hydrolysis of peptide or isopeptide bonds (Komander, Clague and Urbé, 2009). There are approximately 100 DUBs encoded in the human genome which can be classified into six subfamilies based on the structure of their catalytic domains (Clague *et al.*, 2013; Leznicki and Kulathu, 2017). The following five subfamilies are all cysteine proteases; ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Machado-Joseph disease proteases (MJDs; alternatively referred to as Josephins) and the recently identified motif interacting with ubiquitin (MIU)-containing novel DUB family (MINDY). These all possess a triad of conserved amino acid residues (Asp/Asn-His-Cys) that are critical for their catalytic activity. In contrast, a sixth subfamily, termed JAB1/MPN/MOV34 (JAMMs, also known as MPN+) are zinc-dependent metalloproteases (Clague *et al.*, 2013). DUBs can hydrolyse polyubiquitin chains from either the distal or proximal end (exo-cleavage) or within the chain (endo-cleavage). Whether a particular DUB catalyses exo- or endo-cleavage is dependent on the DUB family to which it belongs, the chain linkage type and hence how the DUB recognises polyubiquitin chains (Mevissen and Komander, 2017).

The negative regulation of TGFβ signalling by the inhibitory SMAD proteins includes controlling the activity and stability of TGFβ receptor complexes by ubiquitylation. SMAD7 can directly interact with and hence recruit the E3 ubiquitin-protein ligases SMURF1 and SMURF2 (SMAD ubiquitylation regulatory factor 1 and 2) to the activated TGFβ receptor complexes resulting in their ubiquitin-mediated proteasomal degradation (Kavsak *et al.*, 2000; Ebisawa *et al.*, 2001). SMURF1 and SMURF2 belong to the neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) family of homologous to the E6AP carboxyl-terminus (HECT) E3 ubiquitin ligases and are characterised by the presence of an amino-terminal C2 domain, multiple tryptophan-tryptophan (WW) domains (SMURF1 and SMURF2 contain two and three WW domains respectively) and a carboxy-terminal catalytic HECT domain (Rotin and Kumar, 2009; Morreale and Walden, 2016). Structural analysis has revealed that SMAD7 constitutively associates with SMURF1 and SMURF2 and that this interaction is dependent on the binding of WW protein domains of SMURF1/2 (WW2 and WW3 domains respectively) to the proline-rich PY (Pro-Pro-Xxx-Tyr) motif present within the linker region of SMAD7 (Aragón *et al.*, 2012). Furthermore, the HECT E3 ubiquitin-protein

ligase ITCH (alternatively referred to as atrophin-1-interacting protein 4, AIP4), which also belongs to the NEDD4 family (Morreale and Walden, 2016), has also been reported to inhibit TGFβ signalling (Lallemand *et al.*, 2005). However, unlike SMURF1 and SMURF2, this appears to be via a ubiquitylation-independent mechanism. Although ITCH can induce the ubiquitylation and proteasomal degradation of SMAD7, it also unexpectedly inhibited TGFβ signalling without affecting the expression levels of either the receptors or R-SMADs. It appears that the inhibitory function occurs as a result of the ability of ITCH to stabilise the interaction between SMAD7 and type I TGFβ receptor.

The process of protein ubiquitylation is reversible and therefore a number of deubiquitylating (DUB) enzymes have been identified that mediate the removal of polyubiquitin chains from the type I TGFβ receptor and thus antagonise the function of the E3 ubiquitin ligases discussed previously. Three DUBs belonging to the USP family, USP4, USP11 and USP15 were independently discovered as DUBs that target the type I TGFβ receptor for deubiquitylation using contrasting experimental approaches (Herhaus and Sapkota, 2014). In a genome-wide gain-of-function screen using a SMAD3-SMAD4-dependent transcriptional luciferase reporter system, USP4 was identified as an important regulatory component of TGFβ signalling (Zhang *et al.*, 2012). USP4 directly interacts and deubiquitylates the type I receptor, thereby stabilising receptor levels at the cell membrane. Overexpression of wild-type USP4, but not a catalytically inactive mutant form, enhanced the ability of breast cancer cells to undergo TGFβ-induced EMT. Furthermore, it was found that USP4 is subject to phosphorylation at serine 445 by the serine-threonine protein kinase AKT (also knowns as protein kinase B, PKB). This AKT-mediated phosphorylation at S445 results in redistribution of USP4 from the nucleus to the cytoplasm and cell membrane, as well as affecting its stability and ability to deubiquitylate the type I receptor (Zhang *et al.*, 2012). Thus, USP4 may serve as an important intersection for crosstalk between the TGFβ and the PI3K/AKT signalling pathways.

By contrast, USP11 was identified as a specific interactor of SMAD7 in a proteomic analysis and subsequently revealed as another DUB that regulates TGFβ signalling (Al-Salihi *et al.*, 2012). USP11 directly interacted with SMAD7 but not the R-SMADs SMAD1 and SMAD3 or co-SMAD SMAD4. Similar to USP4, USP11 is able to interact with and deubiquitylate the type I receptor, thereby preventing its proteasomal degradation. Overexpression of USP11 enhanced TGFβ-responsive luciferase reporter activity whereas RNAi-mediated depletion

suppressed the transcription of the TGFβ target genes plasminogen activator inhibitor 1 (PAI-1) and growth arrest and DNA damage-inducible protein 45 (GADD45B). Consistent with this, depletion of USP11 also inhibited the ability of murine mammary epithelial cells to undergo TGFβ-induced EMT (Al-Salihi *et al.*, 2012).

USP15 was identified in an RNAi loss-of-function screen as a DUB whose depletion resulted in the suppression of a TGFβ-responsive luciferase transcriptional reporter (Eichhorn *et al.*, 2012). Modulation of USP15 levels via RNAi-mediated depletion or overexpression resulted in reduced or enhanced phosphorylation of SMAD2 respectively, without affecting the total levels of either SMAD2 or SMAD4. Furthermore, it was found that USP15 forms a complex with SMAD7 and the E3 ubiquitin ligase SMURF2 and is thus also recruited to the type I receptor, where it facilitates the deubiquitylation and hence stability of the type I receptor. Furthermore, in patient-derived glioblastoma neurospheres which exhibited aberrantly high levels of USP15, short hairpin RNA (shRNA)-mediated USP15 depletion suppressed the oncogenic potential of the neurospheres when injected into immunocompromised mice, thus implicating a role for USP15-dependent regulation of TGFβ signalling in glioblastoma pathogenesis. Subsequent research has elucidated additional catalytic functions of USP15 in the context of TGFβ pathway regulation. In addition to directly targeting the type I receptor for deubiquitylation, USP15 can also directly deubiquitylate the SMURF2 E3 ubiquitin ligase and this appears to be an important mechanism to modulate the catalytic activity of SMURF2 (Iyengar *et al.*, 2015). Using a proteomic approach, the authors identified multiple lysine residues within SMURF2 which were deubiquitylated by USP15 and further analysis revealed that lysine 734 (K734), located within the carboxy-terminal catalytic HECT domain, was critical for SMURF2 function. This was demonstrated by the observation that overexpression of K734R mutant form of SMURF2 substantially impeded the ability of SMURF2 to downregulate the type I receptor. Therefore, ubiquitylation of SMURF2 at lysine 734 is required for the ubiquitylation and subsequent proteasomal degradation of the type I TGFβ receptor and USP15-mediated deubiquitylation at this residue inhibits the catalytic activity of SMURF2 and promotes stability of the type I receptor (Iyengar *et al.*, 2015).

In addition to the three USP family members discussed above, the DUB UCH37 (also knowns as UCHL5) has also been reported to regulate TGFβ receptor ubiquitylation (Wicks *et al.*, 2006; Herhaus and Sapkota, 2014). The UCH subfamily of DUBs comprises four members including UCHL1, UCHL3, UCH37 (UCHL5) and breast cancer type I susceptibility protein

(BRCA1)-associated protein 1 (BAP1). UCH family DUBs have been described as ubiquitin chain trimmers and UCH37 itself has been initially characterised as a proteasome-associated DUB that functions to remove ubiquitin moieties from substrates that are destined for proteasomal degradation (Lee *et al.*, 2011). However, more recent research has expanded the physiological functions of the UCH family members (Leznicki and Kulathu, 2017) and consequently highlighted important roles in tumorigenesis and metastasis (Fang and Shen, 2017). UCH37 has been implicated in regulating both TGFβ receptors and R-SMADs (Wicks *et al.*, 2006; Nan *et al.*, 2016). UCH37 was found to interact with SMAD7 and also with SMAD2 and SMAD3 albeit with substantially lower affinity (Wicks *et al.*, 2005). The interaction with SMAD7 facilitates recruitment to the activated receptor complex and enables UCH37 to deubiquitylate and thus enhance the stability of the type I receptor. In addition, overexpression of UCH37 is sufficient to enhance TGFβ-dependent transcriptional luciferase reporter and moreover, expression of UCH37 also attenuates SMURF2-mediated inhibition of TGFβ transcriptional responses (Wicks *et al.*, 2005). Furthermore, inducible shRNA-mediated depletion of UCH37 results in suppression of certain TGFβ-dependent early response genes, and although UCH37 depletion does not appear to affect TGFβ-induced cell proliferation or EMT, it does prevent TGFβ-induced cell migration (Cutts *et al.*, 2011).

1.2.4 Regulation of SMADs by reversible protein phosphorylation

As discussed in section 1.2.3, the phosphorylation of two serine residues with the carboxy-terminal SSXS motif of R-SMADs by the activated type I receptor serine-threonine kinase is a fundamental event in the transduction of TGFβ signals. Therefore, the dephosphorylation of these residues by protein phosphatases represents an effective method of maintaining an appropriately balanced TGFβ signalling response (Bruce and Sapkota, 2012). However, due in part to the understudied nature of protein phosphatases compared with protein kinases, the knowledge of phosphatases responsible for dephosphorylation of R-SMAD SSXS motif remains somewhat limited.

Protein phosphatase 1A (PPM1A; also known as protein phosphatase 2C isoform alpha, PP2Cα) was the first reported SMAD2/SMAD3 C-terminal phosphatase (Lin *et al.*, 2006). PPM1A is a Mg^{2+}/Mn^{2+}-dependent protein serine-threonine phosphatase (PS/TP) that

was observed to be predominantly localised in the nucleus where it directly interacts with both SMAD2 and SMAD3. Overexpression or shRNA-mediated depletion of PPM1A resulted in suppression or enhancement of SMAD2/SMAD3 C-terminal phosphorylation respectively. Moreover, PPM1A depletion increased transcription of the cyclin-dependent kinase (CDK) inhibitors $p15^{INK4B}$ and p21 in response to TGFβ stimulation and thereby enhanced the TGFβ-induced cytostatic response in human immortalised keratinocyte cells. However, subsequent research observed that endogenous PPM1A localised exclusively to cytoplasmic fractions in multiple human and murine cell lines (Bruce *et al.*, 2012), thus contradicting the apparent nuclear localisation of PPM1A reported in the previous study. Furthermore, the cytoplasmic localisation of PPM1A remained unaffected by TGFβ stimulation. This indicates that the identification of a legitimate nuclear-localised protein phosphatase responsible for reversing type I receptor mediated phosphorylation of SMAD2/SMAD3 C-terminal residues remains to be elucidated.

In the same study mentioned above, the serine/threonine-specific protein phosphatase 5 (PP5) was identified in a proteomic screen as an interactor of SMAD2/SMAD3. Moreover, the interaction between PP5 and SMAD2 and SMAD3 was confirmed at the endogenous level and overexpression of PP5 resulted in enhanced SMAD3 C-terminal dephosphorylation. In mouse embryonic fibroblasts (MEFs) derived from $PP5^{-/-}$ mice, SMAD3 phosphorylation was higher compared with wild-type control MEFs following stimulation with TGFβ stimulation. However, this may be accounted for by higher levels of total SMAD3 protein observed in PP5-/- MEFs. Although the messenger RNA (mRNA) expression of SMAD3 was not affected by PP5 deletion, the catalytic activity of PP5 appears to be required for modulation of SMAD3 protein expression (Bruce *et al.*, 2012).

As previously discussed in section 1.2.3, R-SMAD proteins have a globular structure comprised of an amino-terminal MH1 domain and carboxy-terminal MH2 domain connected via a flexible divergent linker region (Massagué, Seoane and Wotton, 2005). This linker region contains multiple residues that are subject to phosphorylation by proline-directed protein kinases which can modulate the localisation, transcriptional ability and stability of R-SMADs. Therefore, the linker region functions to integrate regulatory inputs from multiple signalling pathways that ultimately influence the TGFβ cellular response (Bruce and Sapkota, 2012).

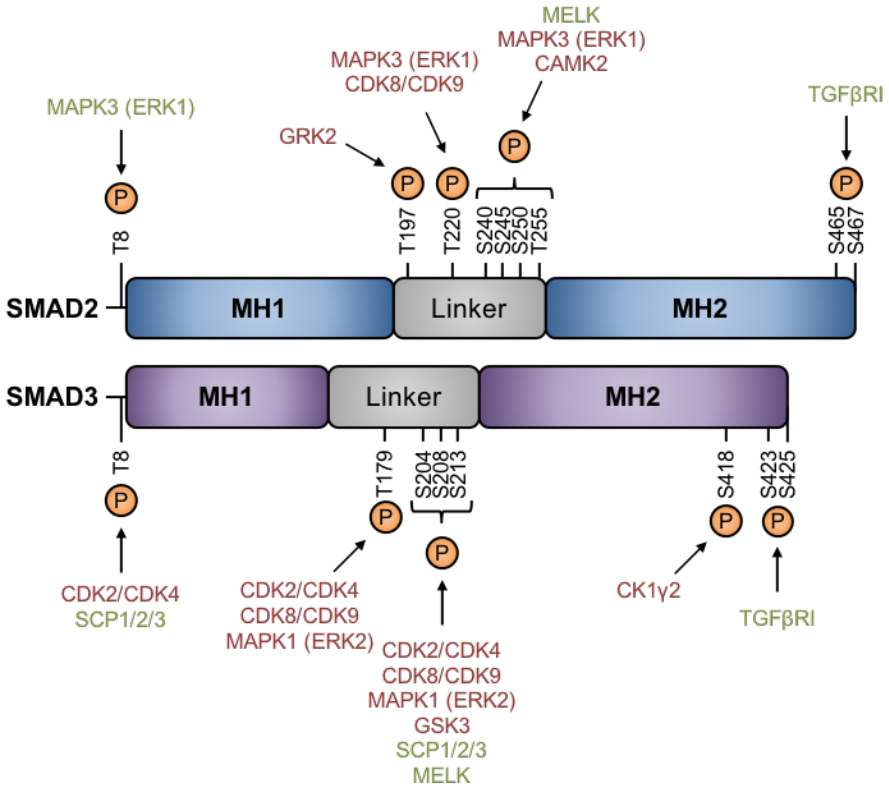


Figure 1E. The phospho-regulation of TGFβ-activated SMAD2 and SMAD3
Schematic diagram of SMAD2 and SMAD3 indicating the principal sites of regulatory phosphorylation and the relevant protein kinases and protein phosphatases responsible. The majority of phosphorylation sites are located within the linker region between the MH1 DNA-binding domain and the MH2 protein-protein interaction domain. Protein kinases which promote SMAD-dependent signalling are indicated in green whereas kinases which attenuate SMAD function are denoted in red. Abbreviations: calcium/calmodulin-dependent protein kinase 2 (CAMK2); casein kinase 1 isoform gamma-2 (CK1γ2); cyclin-dependent kinase (CDK); glycogen synthase kinase 3 (GSK3); β-adrenergic receptor kinase 1 (also referred to as G-protein coupled receptor kinase 2, GRK2); mitogen-activated protein kinase (MAPK; also referred to as extracellular signal-regulated kinase, ERK); maternal embryonic leucine zipper kinase (MELK; also referred to as murine protein serine-threonine kinase 38, MPK38); small carboxy-terminal domain phosphatases, SCPs).

At present, the only protein phosphatases identified to dephosphorylate serine/threonine residues within the linker region of SMAD2 and SMAD3 are the small C-terminal domain phosphatases (SCPs) 1-3 (alternatively termed carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase, CTDSP) (Sapkota *et al.*, 2006; Wrighton *et al.*, 2006). RNAi-mediated depletion of SCP1 and SCP2 enhances the linker phosphorylation of SMAD2 in response to TGFβ stimulation, resulting in decreased TGFβ-dependent gene

transcription (Sapkota *et al.*, 2006). This observation is consistent with the notion that SMAD2/SMAD3 linker phosphorylation functions as an inhibitory mechanism. However, further research is required in order to dissect the functional consequences of proline-directed phosphorylation/dephosphorylation of specific serine/threonine residues within the linker region (Bruce and Sapkota, 2012).

1.2.5 Regulation of SMADs by reversible protein ubiquitylation

As discussed in the preceding section, the linker region of R-SMAD proteins is subject to sequential regulatory phosphorylation events which, in addition to forming high affinity binding sites for transcriptional partners, also mediates interaction with E3 ubiquitin-protein ligases and thus targets the R-SMADs for proteasomal degradation. In the case of SMAD3, phosphorylation of residues within its linker region promotes binding to the transcriptional partner peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1) and the HECT E3 ubiquitin ligase NEDD4L, which binds to the SMAD3 PY motif via its WW protein-protein interaction domain (Kuratomi *et al.*, 2005; Gao *et al.*, 2009; Aragón *et al.*, 2011). Furthermore, the HECT E3 ubiquitin ligases SMURF2 and NEDD4-like E3 ubiquitin-protein ligase WWP1 (also known as TGIF-interacting ubiquitin ligase 1, Tiul1) have also been reported to catalyse the ubiquitylation and subsequent proteasomal degradation of SMAD2 and SMAD3 (Lin, Liang and Feng, 2000; Seo *et al.*, 2004).

Although several E3 ubiquitin ligases have been identified that facilitate the polyubiquitylation and subsequent proteasomal degradation of R-SMADs, to date no deubiquitylating enzymes have been identified that reverse this process and hence prevent R-SMAD degradation. However, prior to the elucidation of its role in the deubiquitylation of the type I TGFβ receptors and the E3 ubiquitin ligase SMURF2, USP15 was reported to interact with and deubiquitylate monoubiquitylated R-SMADs (Inui *et al.*, 2011). Using a loss-of-function siRNA screen, USP15 was identified as a DUB required for TGFβ signalling. Although siRNA-mediated depletion did not appear to affect SMAD3 phosphorylation, it did enhance the mono- and diubiquitylation of SMAD3. The monoubiquitylation sites were mapped and found to be predominantly located within the MH1 domain of SMAD3. In particular, the lysine residue at position 81 appeared to be critically important as mutation to an arginine residue (K81R) resulted in a pronounced attenuation of SMAD3 monoubiquitylation levels. As

previously discussed in section 1.2.3, the MH1 domain of R-SMADs participates in DNA-binding (Massagué, Seoane and Wotton, 2005) and the K81 residue is positioned in close proximity to the highly conserved β-hairpin structure that contacts the major groove of DNA. *In silico* modelling of K81 monoubiquitylated SMAD3 revealed that it is incompatible with DNA recognition and this was subsequently confirmed experimentally using DNA pulldown assays (Inui *et al.*, 2011). Therefore, monoubiquitylation of R-SMADs within their MH1 domain impedes their DNA-binding ability and thus prevents their association with the promoter regions of target genes. However, USP15-mediated removal of this monoubiquitylation promotes the ability of R-SMADs to bind to DNA and hence induce the transcription of target genes (Dupont, Inui and Newfeld, 2012).

More recent research has identified the involvement of a fourth member of the USP subfamily of DUBs in the regulation of TGFβ signalling. USP26 has been reported as a DUB that can reverse the K48-linked polyubiquitylation of the inhibitory SMAD7, thereby enhancing its stability and promoting the downregulation of the type I receptor (Kit Leng Lui *et al.*, 2017). Overexpression of wild type USP26, but not a catalytically inactive form USP26 C/A, enhanced the degradation of the type I receptor, whereas USP26 depletion promoted the downregulation of SMAD7, thereby decreasing receptor ubiquitylation and promoting its stability. Moreover, a significant negative correlation was observed between USP26 expression and SMAD2 phosphorylation in glioblastoma patients, and patients exhibiting lower USP26 levels had reduced overall survival compared with patients expressing high levels of USP26. This indicates that USP26 loss enhances TGFβ signalling and confers a poor prognosis in glioblastoma patients (Kit Leng Lui *et al.*, 2017).

Research has also revealed a role for OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1), a cysteine protease that belongs to the OTU family of deubiquitylating enzymes, in the regulation of TGFβ signalling (Herhaus *et al.*, 2013). A proteomic screen identified OTUB1 as a novel interactor of SMAD3 and subsequent co-immunoprecipitation analysis revealed that it interacts with TGFβ-activated SMAD2/SMAD3-SMAD4 complex and that this interaction is dependent on phosphorylation of SMAD2/SMAD3. RNAi-mediated depletion of OTUB1 suppressed TGFβ-induced transcription of the target genes PAI-1 and connective tissue growth factor (CTGF) however it was found OTUB1 is unable to directly deubiquitylate polyubiquitylated SMAD3 in vitro. Instead, OTUB1 prevents the ubiquitylation of SMAD3 by interacting with and inhibiting the E2 ubiquitin-

conjugating enzymes UBE2D1 and UBE2N. Therefore, OTUB1 can prevent the proteasomal degradation of SMAD3 by inhibiting the efficient transfer of ubiquitin moieties from E2 ubiquitin-conjugating enzymes to E3 ubiquitin-ligating enzymes and thus functions to enhance TGFβ using a mechanism that is independent of its catalytic activity (Herhaus *et al.*, 2013; Herhaus and Sapkota, 2014).

1.3 CELLULAR FUNCTIONS OF TGFβ FAMILY CYTOKINES

1.3.1 The importance of cellular context

Since the discovery and characterisation of TGFβ cytokines during the 1980s, research conducted over the following decades has defined the core molecular components of the pathway as well as identifying ancillary components and regulators that function to modulate the signalling pathway. However, it became evident that TGFβ can exert different, sometimes opposite, effects depending on cell type and context (Massagué, 2012a). Consequently, the question of how a conceptually simple ligand-receptor-SMAD signal transduction pathway could regulate a diverse array of cellular processes remained somewhat enigmatic.

More recent and current research has made considerable progress towards understanding how context determines the nature of the cellular response to TGFβ signals. These contextual determinants that define the TGFβ response can be broadly classified into three types, which are often interconnected; those which function at the level of signal transduction, the factors which co-operate with SMADs to regulate target gene transcription and those which define the epigenetic status of the cell.

The TGFβ signal transduction pathway is subject to both extracellular and intracellular regulation which contributes to determining the context-dependent cellular response to TGFβ signals. Examples include the abundance and activity of different TGFβ family members, the presence of ligand traps that regulate the access of ligands to cell-surface receptors and facilitate the formation of ligand gradients (particularly important during embryonic development), the combinations of type I and type II receptors and requirement for co-receptors, proteins which regulate the stability of receptors and SMADs and the influence of cross-talk from other signalling pathways.

At the level of transcriptional regulation, a unifying paradigm has been revealed involving collaboration between SMADs, signal-driven transcription factors (SDTFs) that are directly activated by TGFβ receptors and lineage-determining transcription factors (LDTFs) which differ depending on cell type (David and Massagué, 2018). The co-operation of LDTFs with SMADs determines the binding of activated SMAD complexes to specific genomic loci and whether the expression of target genes will be either positively or negatively regulated.

The epigenetic status of a cell dictates whether the chromatin conformation is in an 'open' permissive state in which the promoter regions of target genes are accessible to TGFβ-activated SMAD complexes or in a 'closed' repressive state which is not accessible for transcriptional regulation. The epigenetic status is particularly important in the context of stem cell pluripotency and differentiation. Under conditions which promote self-renewal of embryonic stem cells (ESCs), genes required for pluripotency are in an open conformation that permits transcriptional activation in response to TGFβ signals, whereas genes involved in differentiation remain in a repressive chromatin conformation and are thus refractory to these signals (Massagué, 2012a). The involvement of TGFβ in regulating stem cell pluripotency and differentiation is discussed in more detail in section 1.4.3.

1.3.2 Immune regulation

Members of the TGFβ family of cytokines have critical and pleiotropic immunoregulatory functions. $TGF\beta_1$ is the predominant member expressed in the immune system and can function in both autocrine and paracrine modes to control the differentiation, proliferation and activation of leukocyte lineage cells including lymphocytes, macrophages and dendritic cells (Letterio and Roberts, 1998). Disruption of TGFβ signalling components in mice leads to the development of numerous immunopathological phenotypes, providing further evidence of the importance of TGFβ signalling in the regulation of immune responses (Chen and ten Dijke, 2016).

The pleiotropic nature of TGFβ action in the immune system is particularly evident in the regulation of T lymphocytes, and it has become apparent that TGFβ is a critical regulator of thymic T lymphocyte development in addition to contributing to peripheral T lymphocyte homeostasis (Gorelik and Flavell, 2002; Li and Flavell, 2008; Tu, Chia and Chen, 2014). The important function of TGFβ in regulating the specification of various T lymphocyte subtypes is predominantly mediated via the cooperation of SMADs with different lineage-determining transcription factors (LDTFs) (David and Massagué, 2018). For example, TGFβ can promote the differentiation of naive $CD4^+$ (T-cell surface glycoprotein CD4) T cells into regulatory T (T_{reg}) cells in peripheral lymphoid organs (Yamagiwa *et al.*, 2001; Zheng *et al.*, 2004). $CD4^+$ T_{reg} cells develop from naive $CD4^+$ T cells in both the thymus and periphery and participate in the maintenance of immunological self-tolerance. One of the principal functions of $CD4^+$ T_{reg} cells

in the periphery is to suppress the activation and proliferation of potentially harmful self-reactive T cells that have evaded thymic clonal deletion (Josefowicz, Lu and Rudensky, 2012). The lineage-determining transcription factor forkhead box protein P3 (FOXP3) is a principal determinant of the $CD4^+$ T_{reg} cell phenotype (Hori, Nomura and Sakaguchi, 2003) and the induction of FOXP3 expression in peripheral $CD4^+$ naive T cells is predominantly mediated by TGFβ signalling (Chen *et al.*, 2003; Selvaraj and Geiger, 2007). Subsequent research demonstrated that TGFβ-mediated regulation of peripheral T_{reg} cell differentiation requires cooperation with other signal-driven transcription factors (SDTFs). It was demonstrated that TGFβ-activated SMAD3 and the transcription factor nuclear factor of activated T-cells (NFAT) bind to conserved sequences within a 5' enhancer element for the *FOXP3* gene (Mantel *et al.*, 2006; Tone *et al.*, 2008). Furthermore, the binding of both SMAD3 and NFAT to the *FOXP3* enhancer is required for histone acetylation of the enhancer region and concomitant induction of FOXP3 expression (Tone *et al.*, 2008).

Research has also indicated a critical requirement for interleukin-2 (IL-2) signalling in the TGFβ-mediated induction of FOXP3 expression that results in the differentiation of peripheral $CD4^+$ naive T cells into $CD4^+$ T_{reg} cells (Josefowicz, Lu and Rudensky, 2012). This is demonstrated by the observation that TGFβ stimulation is unable to induce the expression of FOXP3 in peripheral $CD4^+$ naive T cells derived from IL-2-deficient mice. However, treatment with exogenous IL-2, but not IL-7 or IL-15 (cytokines which signal through IL-2 receptor subunit gamma (IL-2Rγ) and IL-2Rγ and IL-2Rβ subunits respectively), was sufficient to restore the ability of TGFβ to induce FOXP3 expression (Davidson *et al.*, 2007; Zheng *et al.*, 2007).

1.3.3 Cellular proliferation

TGFβ cytokines were initially identified in crude acid-ethanol extracts from sarcoma and carcinoma-derived cell lines and were found to be acid-stable, soluble polypeptides with the ability to stimulate the growth of normal (*i.e.* untransformed) rat renal fibroblasts (Roberts *et al.*, 1980), hence their designation as 'transforming growth factors.' However, subsequent research using purified TGFβ demonstrated that it could also elicit strong anti-proliferative effects in certain cell types (Roberts *et al.*, 1985). Research over the following three decades has revealed that TGFβ is a multifunctional cytokine that can regulate diverse cellular

processes including cellular proliferation and differentiation, EMT, cell death and modulation of the cell microenvironment and that TGFβ signalling is highly dependent on cellular context. The cytostatic effect of TGFβ has been widely investigated due to its major contribution to the tumour suppressive function of TGFβ (Massagué, 2008a) and represents a paradigm of TGFβ-mediated cellular response.

The molecular mechanisms underlying the cytostatic effect of TGFβ were predominantly delineated using epithelial cell types, however the ability of TGFβ to negatively regulate cellular proliferation has been observed in multiple cells types including endothelial, neuronal and cells of the immune system (Siegel and Massagué, 2003). The eukaryotic cell cycle is divided into four sequential phases: G_1 phase, S (DNA synthesis) phase, G_2 phase and M (mitosis) phase. Early research indicated that TGFβ induces cell-cycle arrest at the G_1 phase (Laiho *et al.*, 1990; Geng and Weinberg, 1993; Polyak, Kato, *et al.*, 1994) and this research contributed to the identification of the restriction (R)-point transition that occurs in mid-late G_1 phase (Planas-Silva and Weinberg, 1997). The R-point represents a specific period during which cells perceive and interpret extracellular signals and decide whether or not to proceed through the cell cycle. Once a cell progresses through the R-point transition, it is committed to completing the remainder of the cell cycle and is no longer competent to regulation by extracellular signals. Thus, the addition of TGFβ to cells which have progressed through the R-point transition does not inhibit the ongoing cell cycle.

The cytostatic response exerted by TGFβ on epithelial cells involves the regulation of two distinct classes of cell-cycle regulators that both function to promote cell proliferation; the repression of certain growth-promoting transcription factors and the induction of cyclin-dependent kinase (CDK) inhibitors. Transcriptomic profiling of the TGFβ-mediated cytostatic response in three epithelial cell lines derived from different human tissues revealed the minimal general features of the cytostatic programme, involving the up-regulation of the CDK inhibitors $p15^{INK4B}$ (also termed cyclin-dependent kinase 4 inhibitor B, *CDKN2B*) and $p21^{CIP1}$ (also termed cyclin-dependent kinase inhibitor 1A, *CDKN1A*) and the repression of the transcription factors c-MYC, ID-1, ID-2 and ID-3 (Kang, Chen and Massagué, 2003).

Progression through the cell cycle is controlled by the actions of cyclin-dependent kinases (CDKs). CDKs are the catalytic subunits of heterodimeric serine-threonine protein kinases which require the binding of a regulatory subunit (termed cyclin) as well as phosphorylation of a threonine residue within the activation loop of the kinase subunit in

order to be completely active (Hochegger, Takeda and Hunt, 2008). The cyclin D-dependent kinases CDK4 and CDK6 control progression through the G_1 phase of the cell cycle by phosphorylating retinoblastoma protein (pRb; also known as p105), retinoblastoma-like protein 1 (RBL1; also known as p107) and retinoblastoma-like protein 2 (RBL2; also known as p130) (Malumbres and Barbacid, 2005). The cyclin D-CDK4 and cyclin D-CDK6 complexes are both specifically inhibited by $p15^{INK4B}$, the expression of which is rapidly induced by TGFβ in epithelial cells (Hannon and Beach, 1994; Reynisdóttir *et al.*, 1995). $p15^{INK4B}$ belongs to the inhibitors of CDK4 (INK4) family of cyclin-dependent kinase inhibitors which also includes $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$ (Malumbres and Barbacid, 2005). The INK4 proteins specifically bind to and inhibit the monomeric CDK4 and CDK6 catalytic subunits, disrupting their catalytic sites and preventing association with D-type cyclins (Sherr and Roberts, 1999). Furthermore, in addition to transcriptional upregulation, it has been observed that TGFβ can also enhance the protein stability of $p15^{INK4B}$ (Sandhu *et al.*, 1997). Along with $p15^{INK4B}$, TGFβ has also been shown to transcriptionally induce a number of other CDK inhibitors, including $p21^{CIP1}$ (Datto, Yu and Wang, 1995; Reynisdóttir *et al.*, 1995), $p27^{KIP1}$ (also known as cyclin-dependent kinase inhibitor 1B, CDKN1B) (Polyak, Kato, *et al.*, 1994a; Polyak, Lee, *et al.*, 1994) and $p57^{KIP2}$ (also known as cyclin-dependent kinase inhibitor 1C, CDKN1C) (Lee, Reynisdóttir and Massagué, 1995; Matsuoka *et al.*, 1995). Collectively, these three CDK inhibitors constitute the CDK interacting protein/kinase inhibitory protein (CIP/KIP) family, which along with the aforementioned INK4 proteins, comprise the two families of mammalian CDK inhibitors involved in regulating the cell cycle. In contrast to the INK4 family members, which specifically interact with and inhibit the cyclin-D-dependent CDKs (CDK4 and CDK6), CIP/KIP family members can interact and inhibit many different cyclin-CDK complexes. However, CIP/KIP proteins display higher affinity for G_1 and S phase CDKs and therefore, along with the INK4 proteins, primarily function to regulate the G_1/S phases of the cell cycle. Subsequent research demonstrated that the TGFβ-mediated transcriptional induction of $p15^{INK4B}$ and $p21^{CIP1}$ expression is dependent on both SMAD and members of the forkhead box O (FOXO) family of transcription factors (FOXO1, FOXO3 and FOXO4) (Seoane *et al.*, 2004; Gomis *et al.*, 2006).

Interestingly, increased levels of $p15^{INK4B}$ in response to TGFβ stimulation also functions to regulate the action of $p27^{KIP1}$. In proliferating cells, $p27^{KIP1}$ is predominantly found in a complex with cyclin-D-CDK4/CDK6 that remains catalytically active and thus functions as a reservoir of $p27^{KIP1}$ (Blain, Montalvo and Massagué, 1997). However, upon increased

expression of $p15^{INK4B}$ in response to TGFβ stimulation, $p15^{INK4B}$ can prevent $p27^{KIP1}$ from binding to cyclin-D-dependent CDK4/CDK6 complexes. This promotes $p27^{KIP1}$ to interact with cyclin-CDK2 complexes, thereby inhibiting the kinase activity of CDK2 (Reynisdóttir and Massagué, 1997). In this way, TGFβ can induce cytostasis at the G_1/S phase by targeting two distinct cyclin-CDK complexes for inhibition through the upregulation of $p15^{INK4B}$ and redistribution of $p27^{KIP1}$. The inhibition of these G_1 phase cyclin-CDK complexes prevents the hyperphosphorylation of pRb (p105), RBL1 (p107), RBL2 (p130) and other substrates, therefore preventing the transactivation of genes required for S phase progression (Siegel and Massagué, 2003).

In addition to the transcriptional induction of CDK inhibitors, the transcriptional repression of growth-promoting transcription factors is another important mechanism by which TGFβ can induce cell-cycle arrest. Principal among these are the proto-oncogenic transcription factor c-MYC and the DNA-binding protein inhibitors (also knowns as inhibitors of differentiation) ID-1, ID-2 and ID3 (Kang, Chen and Massagué, 2003).

TGFβ stimulation has been shown to rapidly down-regulate both the RNA and protein levels of c-MYC in various different cell lines (Alexandrow and Moses, 1995). The importance of this is highlighted by the observation that TGFβ-mediated cell-cycle arrest is prevented in cells in which c-MYC is overexpressed or in which there is a failure to down-regulate c-MYC expression (Alexandrow *et al.*, 1995; Chen, Kang and Massagué, 2001). Research from a number of independent groups identified the components of a transcriptional repressor complex and the molecular mechanism by which it facilitates the down-regulation of c-MYC in response to TGFβ (Chen *et al.*, 2002; Yagi *et al.*, 2002; Frederick *et al.*, 2004). This complex consists of SMAD3, SMAD4, the transcription factor E2F4 and the transcriptional repressor RBL1. The SMAD3-SMAD4-E2F4 transcriptional repressor complex binds to adjacent SMAD and E2F binding sites, referred to as a TGFβ inhibitory element (TIE) within the promoter region of the *c-MYC* gene (Chen *et al.*, 2002). As previously mentioned, RBL1 (alternatively referred to as p107), along with pRb (p105) and RBL2 (p130), which collectively comprise the pocket protein family of cell-cycle regulators (Cobrinik, 2005), are subject to CDK-dependent phosphorylation and inhibition that occurs in the nucleus. However, the fraction of RBL1 that participates in the SMAD3-SMAD4-E2F4 transcriptional repressor complex resides in the cytoplasm and is therefore not subjected to CDK-mediated inhibitory phosphorylation (Siegel and Massagué, 2003). Intriguingly, in addition to preventing its growth-promoting functions,

the repression of c-MYC expression also contributes to the transcriptional activation of the CDK inhibitors $p15^{INK4B}$ and $p21^{CIP1}$ that occurs in response to the same TGFβ signals (discussed previously). Therefore, c-MYC down-regulation serves two functions and represents a critical node at the centre of the TGFβ cytostatic response (Siegel and Massagué, 2003).

The transcriptional repression of c-MYC constitutes a hallmark of the cytostatic programme mediated by TGFβ, however the repression of other growth-promoting transcription factors also contributes to this process. TGFβ-induced cell-cycle arrest in epithelial cells involves the suppression of the ID-1, ID-2 and ID-3 proteins (Kang, Chen and Massagué, 2003), which along with ID-4, comprise the inhibitors of DNA binding/differentiation (ID) family of transcription factors. The ID proteins contain a helix-loop-helix (HLH) domain however they lack a DNA-binding domain and they therefore they operate to antagonise the function of basic HLH (bHLH) transcription factors (Ruzinova and Benezra, 2003). TGFβ family members, including BMP isoforms, have been shown to both positively and negatively regulate ID members depending on cell type and context. ID proteins have been reported to promote cell proliferation through a number of different mechanisms. For example, overexpression of ID-1 in cultured cells results in reduced expression of $p16^{INK4A}$ (also known as cyclin-dependent kinase inhibitor 2A, CDKN2A), a member of the INK4 family of CDK inhibitors. $p16^{INK4A}$ can arrest cells in the G_1 phase by inhibiting CDK4 and CDK6 and has been implicated in mediating cellular senescence (*i.e.* irreversible G_0 phase) (Ohtani *et al.*, 2001). Overexpression of ID-1 and the resulting suppression of $p16^{INK4A}$ has also been reported to contribute to increased proliferation of prostate cancer cells (Ouyang *et al.*, 2002). The ability of TGFβ to repress the expression of ID-1 occurs via a 'self-enabling response' and is dependent on the induction of the transcriptional repressor activating transcription factor 3 (ATF3; otherwise referred to as cyclic AMP-dependent transcription factor) (Kang, Chen and Massagué, 2003). In epithelial cells, TGFβ rapidly induces the expression of ATF3 in a SMAD3-dependent manner (the primary gene response). ATF3 subsequently interacts with SMAD3 to form a transcriptional repressor complex, along with SMAD4. The SMAD3-SMAD4-ATF3 complex binds to the ID1 gene promoter, thereby mediating the repression of ID-1 (the secondary gene response).

Furthermore, in certain osteosarcoma and glioblastoma cell lines, ID-2 (but not ID-1 or ID-3) has been shown to directly interact with pRb, RBL1 and RBL2 and inhibit the growth-suppressing activities of these proteins, thereby promoting cell proliferation (Lasorella,

Iavarone and Israel, 1996). Subsequent research highlighted the importance of TGFβ-mediated c-MYC repression in the regulation of ID-2 by demonstrating that ID-2 is a direct transcriptional target of MYC transcription factors. The binding of c-MYC or N-MYC to enhancer (E)-box elements within the *ID2* promoter region induced up-regulation of ID-2 expression (Lasorella *et al.*, 2000). Thus, c-MYC downregulation in response to TGFβ signals may result in the concomitant suppression of ID-2 expression in certain cell types. However, although the initial repression of ID-2 during the TGFβ-mediated cytostatic response is dependent on suppression of c-MYC, sustained repression of ID-2 appears to involve a c-MYC-independent mechanism, as c-MYC levels recover even though TGFβ stimulation is maintained. Research has indicated that the sustained repression of ID-2 expression requires upregulation of the transcriptional repressors MAX-interacting protein 1 (MXI-1) and MAX dimerisation protein 4 (MAD4), which compete with c-MYC for binding to the transcriptional regulator MYC-associated factor X (MAX) (Siegel, Shu and Massagué, 2003). Therefore, prolonged TGFβ stimulation results in a shift from c-MYC-MAX complexes to MXI-1/MAD4-MAX heterodimeric complexes at the *ID2* promoter, facilitating the sustained repression of ID-2 during TGFβ-induced cell-cycle arrest.

The appropriate balance between cell proliferation and cell death is fundamental to correct tissue growth and physiological homeostasis. Consequently, perturbations of this balance can result in development of pathological conditions, most notably tumorigenesis, and the participation of TGFβ signalling will be discussed in subsequent chapters.

1.3.4 Epithelial-mesenchymal transition (EMT)

Epithelial-mesenchymal transition (EMT) is the cellular process by which a polarised epithelial cell undergoes profound biochemical and morphological alterations that enable it to acquire a mesenchymal cell phenotype (Kalluri and Weinberg, 2009). EMT is a fundamental developmental process which occurs multiple times throughout embryogenesis and also participates in the wound healing response. However, it has become apparent that EMT contributes to disease pathogenesis, most notably tissue fibrosis (Kim, Sheppard and Chapman, 2018) and tumour invasion and metastasis (Hanahan and Weinberg, 2011).

Epithelial cells typically form layers, tubes or vesicles in which the cells exhibit apical-basal polarity and intimately associate with adjacent cells through specialised lateral cell-cell

contacts including tight junctions, adherens junctions and desmosomes. Upon initiation of EMT, one of the earliest events to occur is the dissolution of tight junctions (also knowns as occluding junctions) at the apical-lateral surfaces, characterised by decreased expression of claudin and occludin transmembrane proteins and delocalisation of the cytoplasmic protein zonula occludens-1 (ZO-1; also referred to as tight junction protein-1, TJP1) (Lamouille, Xu and Derynck, 2014). This is followed by disassembly of the more basolateral adherens junctions and desmosome complexes, with concomitant reorganisation of the actin cytoskeleton. Consequently, epithelial cells lose their apical-basolateral cell polarity and develop a fibroblastoid (*i.e.* spindle-like) morphology with 'front-rear' polarity. In addition, cells undergoing EMT increase secretion of extracellular proteases and alter expression of extracellular matrix (ECM) proteins. This enables the cells to become migratory and, in the context of tumorigenesis, facilitates invasion and metastasis. Throughout the process of EMT there is a reprogramming of the transcriptional profile of the cell, in which epithelial gene expression is lost and there is enhanced or *de novo* expression of mesenchymal genes such as N-cadherin, vimentin and fibronectin (Zavadil *et al.*, 2001; Valcourt *et al.*, 2005).

TGFβ has been identified as a potent inducer of EMT in both transformed and non-transformed epithelial cell types in culture (Miettinen *et al.*, 1994; Caulin *et al.*, 1995; Piek *et al.*, 1999; Valdés *et al.*, 2002) and numerous studies have demonstrated the importance of TGFβ-activated SMAD-dependent signalling in this process (Xu, Lamouille and Derynck, 2009). One of the principal mechanisms by which TGFβ can induce EMT is by regulating the expression of critical transcription factors that mediate the transcriptional reprogramming that occurs during the process. This transcriptional reprogramming involves three distinct families of transcription factors: Snail, two zinc finger E-box-binding homeobox (ZEB1 and ZEB2) proteins and members of the basic helix-loop-helix (bHLH) family. The Snail superfamily are zinc-finger transcription factors of which there are five encoded in the human genome: SNAI1 and SNAI2 (more commonly referred to as Snail and Slug respectively), SNAI3, SCRATCH1 and SCRATCH (Nieto, 2002). In epithelial cells, both Snail and Slug have been shown to mediate the transcriptional repression of the adhesion protein E-cadherin through interaction with E-box elements within the *CDH1* gene promoter (Batlle *et al.*, 2000; Cano *et al.*, 2000; Bolós *et al.*, 2003). The delocalisation and down-regulation of E-cadherin expression is considered to be a hallmark event of EMT that directly facilitates the dissolution of adherens junctions in addition to contributing to transcriptional reprogramming via the

release of soluble β-catenin (Lamouille, Xu and Derynck, 2014). TGFβ can induce Snail expression in epithelial cells, however this appears dependent on activation of the MAPK pathway, requiring the activity of the MAPKKs MEK1 and MEK2, but independent of SMAD4 (Peinado, Quintanilla and Cano, 2003). Furthermore, TGFβ-mediated induction of Snail has also been reported to upregulate the expression of matrix metalloproteinase 2 and 9 (MMP2 and MMP9) (Jordà *et al.*, 2005; Taki *et al.*, 2006). In epithelial cells, the upregulation of MMP2 and MMP9 in response to TGFβ may involve activation of p38 MAPK signalling but not ERK1/ERK2 (Kim, Kim and Moon, 2004) and contributes to the invasive behaviour of carcinoma cells.

In addition to regulating the transcriptional programme, TGFβ can also induce EMT by promoting the disassembly of cell junctions and concomitant loss of apical-basal cell polarity (Lamouille, Xu and Derynck, 2014). TGFβ has been shown to directly regulate the polarity protein partitioning defective 6 homolog alpha (PAR6) (Ozdamar *et al.*, 2005). PAR6 is a component of the PAR complex, along with PAR3, atypical protein kinase C (aPKC) and the Rho family small GTPase cell division control protein 42 homolog (CDC42) (Joberty *et al.*, 2000; Lin *et al.*, 2000). The PAR complex is believed to be required for the assembly and maintenance of tight junctions (but not adherens junctions), thereby facilitating cell-cell contact and sustaining epithelial cell polarity (Gao 2002). In epithelial cells, the TGFβ type I receptor (TGFβR1) colocalises with PAR6 and ZO-1 at tight junctions, whereas the TGFβ type II receptor (TGFβR2) was found to be uniformly distributed in discrete puncta at the apical surface. However, upon TGFβ stimulation, TGFβR2 redistributed to tight junctions and colocalised with both TGFβR1 and ZO-1. Moreover, TGFβR1 was found to constitutively interact with PAR6 and occludin, a structural component of tight junctions at the endogenous level, and interaction of PAR6 with TGFβR1 facilitated its phosphorylation at Ser345 by the constitutively active TGFβR2 kinase. Mutation of the serine residue to alanine prevented TGFβ-induced dissolution of tight junctions (Ozdamar *et al.*, 2005). TGFβR2-mediated phosphorylation of PAR6 enables recruitment of the E3 ubiquitin-ligase SMURF1 to the PAR complex, where it can regulate cell polarity by mediating the ubiquitylation and subsequent proteasomal degradation of RAS homolog gene family member A (RhoA)(Wang *et al.*, 2003; Tian *et al.*, 2011). RhoA is the prototypical member of the Rho family of small GTPases and has important functions in modulating the formation and stability of cell-cell junctions (Fukata and Kaibuchi, 2001; Sahai and Marshall, 2002; Perez-Moreno, Jamora and Fuchs, 2003).

Therefore, the localised SMURF1-mediated degradation of RhoA promotes TGFβ-dependent dissolution of tight junctions during EMT (Ozdamar *et al.*, 2005).

1.3.5 Apoptosis

Apoptosis is an evolutionary conserved form of regulated cell death that occurs in both physiological and pathophysiological situations in multicellular organisms. The term apoptosis was first used in 1972 to describe a form of programmed cell death that exhibited morphological features distinct from those observed in cells undergoing pathological, necrotic cell death (Hengartner, 2000).

TGFβ can regulate apoptosis in a variety of different cell types and physiological contexts. TGFβ-mediated apoptosis has been shown to play an important role in organogenesis and limb formation during embryonic development or during normal adult tissue remodelling (Dünker, Schmitt and Krieglstein, 2002; Schuster, Dünker and Krieglstein, 2002; Beier *et al.*, 2006). Furthermore, the ability of TGFβ to induce apoptosis is one of the principal contributions to the tumour suppressive function of TGFβ cytokines (Ikushima and Miyazono, 2010a). TGFβ can either promote or suppress apoptosis, although in the majority of cases the pro-apoptotic ability of TGFβ has been reported. Consequently, a coherent molecular understanding of how TGFβ regulates apoptosis has yet to be elucidated, and as with many aspects of TGFβ signalling, it appears to be highly dependent on cell type and context. Furthermore, apoptosis is regulated by many other signals and therefore integration of multiple signalling pathways is likely to be required before a cell decides upon the important decision between survival and programmed cell death (Zhang, Alexander and Wang, 2017).

A number of studies have highlighted the importance of SMAD proteins as mediators of TGFβ-induced apoptosis in multiple different cell types. In human pulmonary epithelial cells, constitutive overexpression of SMAD3 in the presence of TGFβ stimulation strongly induced apoptosis. Although overexpression of SMAD2 could also potentiate TGFβ-induced apoptosis, this occurred to a much lesser degree (Yanagisawa *et al.*, 1998). Furthermore, overexpression of a dominant negative form of SMAD3, in which the three serine residues within the carboxy-terminal SSXS motif were mutated to alanine, or the inhibitory SMAD7

impaired TGFβ-induced apoptosis in human hepatocellular carcinoma cells (Yamamura *et al.*, 2000).

Activated SMAD transcriptional complexes have been shown to regulate the expression of several apoptotic genes. The TGFβ-inducible early-response gene (TIEG1; also known as Krueppel-like factor 10, KLF10) was one of the first TGFβ-responsive genes identified that contributes to TGFβ-induced apoptosis. TIEG1 is a zinc-finger transcription factor that is transcriptionally induced by TGFβ in various pancreatic epithelial cells (Tachibana *et al.*, 1997) and pulmonary epithelial cells (Chalaux *et al.*, 1999). Overexpression of TIEG1 inhibits cell proliferation and simultaneously induces apoptosis in TGFβ-responsive pancreatic epithelial cells (Tachibana *et al.*, 1997). Furthermore, the pro-apoptotic function of TIEG1 may be due in part to its ability to supress the expression of the apoptosis regulator B-cell lymphoma 2 (BCL-2) protein. BCL-2 is the prototypical member of the evolutionarily conserved BCL-2 protein family which regulates apoptosis through their ability to regulate mitochondrial cytochrome c release (*i.e.* the intrinsic apoptotic pathway) (Czabotar *et al.*, 2014). The BCL-2 family comprises three subfamilies; the anti-apoptotic (pro-survival) subfamily, the pro-apoptotic subfamily and the BH3-only subfamily. All the family members share sequence and structural homology and contain between one and four BCL-2 homology (BH) domains (BH1-BH4). Additionally, most members also contain a transmembrane (TM) domain, which enables association with organelle membranes, most notably the outer mitochondrial membrane. In humans, there are six members of the anti-apoptotic subfamily, which is comprised of BCL-2 along with BCL-XL (also known as BCL-2-like protein 1, BCL2-L1), BCL-W (also known as BCL-2-like protein 2, BCL2-L2), induced myeloid leukaemia cell differentiation protein (MCL1; also known as BCL-2-like protein 3, BCL2-L3), BCL2-A1 (BCL-2-like protein 5, BCL2-L5) and BCL-B (also known as BCL-2-like protein 10). The members of this subfamily contain four BH domains and a TM domain, with the exception of BCL2-A1 which lacks a TM domain. The pro-apoptotic subfamily consists of three members, BAX (also known as BCL-2-like protein 4, BCL2-L4), BCL-2 homologous antagonist/killer (BAK; also known as BCL-2-like protein 7, BCL2-L7) and BCL-2-related ovarian killer protein (BOK; also known as BCL-2-like protein 9, BCL2-L9). As with the anti-apoptotic subfamily members, the pro-apoptotic members also contain four BH domains and a TM domain and adopt similar globular structures. They promote apoptosis by undergoing oligomerisation to form pores in the outer mitochondrial membrane, resulting in efflux of cytochrome c into the cytosol and ultimately

activation of the executioner caspase proteases caspase-3 and caspase-7 (Tait and Green, 2010). The third subfamily of BCL-2 proteins are the BH3-only proteins, of which there are eight mammalian members consisting of BH3-interacting domain death agonist (BID), BCL2-associated agonist of cell death (BAD; also knowns as BCL2-L8), BCL2-interacting mediator of cell death (BIM; also known as BCL2-L11), BCL2-interacting killer (BIK), BCL2-modifying factor (BMF), activator of apoptosis harakiri (HRK; also known as BH3-interacting domain-containing protein 3, BID3), NOXA (also known as phorbol-12-myristate-13-acetate-induced protein 1, PMAIP1) and p53 upregulated modulator of apoptosis (PUMA; also known as BCL2-binding component 3, BBC3). Unlike the other two BCL-2 protein subfamilies, the BH3-only members display little sequence homology and (as their name implies) only contain a BH3 domain. Overexpression of any of the BH3-only proteins is sufficient to promote apoptosis, however the members are differentially regulated (Taylor, Cullen and Martin, 2008).

TGFβ has been shown to induce apoptosis by regulating the expression of a number of different BCL-2 protein family members in various different cell types. For example, in both liver and mammary gland epithelial cells, TGFβ stimulation results in apoptosis and the upregulation of the BH3-only proteins BIM and BMF in a SMAD4-dependent manner. RNAi-mediated depletion of either BIM or BMF protected cells from TGFβ-induced cell death and this protection was further enhanced when BIM and BMF protein levels were depleted simultaneously (Ramjaun *et al.*, 2007). Similarly, in a gastric epithelial cell line which is amenable to TGFβ-mediated apoptosis, BIM and BMF expression was upregulated in response to TGFβ stimulation. However, in this cell context, only RNAi-mediated depletion of BIM expression was able to render the cells resistant to TGFβ-induced apoptosis (Ohgushi *et al.*, 2005). Furthermore, TGFβ can induce apoptosis in human Burkitt lymphoma (BL) cell lines by mediating the differential transcriptional regulation of BCL-2 family members. Stimulation of BL cells with TGFβ promoted apoptosis by enhancing the transcription of the pro-apoptotic BH3-only subfamily member BIK, while simultaneously repressing the expression of the anti-apoptotic subfamily member BCL-XL (Spender *et al.*, 2009). The transcriptional repression of BIK involves the TGFβ-dependent recruitment of SMAD3 and SMAD4 to the endogenous BIK promoter.

In certain hepatocellular carcinoma cell lines, the expression of death-associated protein kinase 1 (DAPK1) is increased during TGFβ-induced apoptosis (Jang *et al.*, 2002). DAPK1 is a calcium/calmodulin (Ca^{2+}/CaM)-dependent serine-threonine protein kinase that

has been reported to mediate apoptosis in response to numerous different stimuli. One of the proposed mechanisms by which DAPK1 is thought to mediate apoptosis is through activation of the tumour suppressor cellular tumour antigen p53 (TP53) in a p19ARF-dependent manner (Raveh *et al.*, 2001). The regulation of DAPK1 transcription by TGFβ is dependent on SMAD2 and SMAD3, and expression of a kinase-inactive mutant form of DAPK1 or downregulation of DAPK1 expression using RNAi inhibited TGFβ-induced apoptosis (Jang *et al.*, 2002).

In addition to transcriptional regulation, a number of studies have identified proteins whose expression, although not regulated by TGFβ, is required for TGFβ-induced apoptosis. For example, the adaptor protein DAXX (also knowns as death domain-associated protein 6, DAP6) has been reported to directly interact with the TGFβ type II receptor and mediate TGFβ-induced apoptosis in murine hepatocytes. DAXX has previously been shown to interact with the death domain of the transmembrane cell death receptor tumour necrosis factor (TNF) receptor superfamily member 6 (also known as Fas) to activate the c-Jun amino-terminal kinase (JNK) signalling pathway and potentiate Fas-induced apoptosis (Yang *et al.*, 1997). In addition, DAXX has also been reported to interact with the cytoplasmic domain of the TGFβ type II receptor kinase and in doing so functions as an adaptor protein to facilitate TGFβ-induced activation of the JNK pathway and therefore mediate TGFβ-induced apoptosis (Perlman *et al.*, 2001).

1.4 TGFβ SIGNALLING IN HUMAN PATHOLOGIES

1.4.1 TGFβ signalling in cancer

TGFβ cytokines regulate cytostasis and apoptosis, which are fundamental cellular processes. These processes, along with regulation of cell adhesion and the cellular microenvironment, contribute to the tumour suppressive effects exerted by TGFβ in normal, premalignant cells. However, perturbations in the signalling pathway, either via inactivation of core components or defective downstream responses (*i.e.* loss of tumour suppressive functions), can enable cancer cells to evade the tumour suppressive influence of TGFβ. Furthermore, TGFβ signalling can be co-opted by cancer cells to facilitate cancer progression and metastasis via the ability of TGFβ to promote epithelial-mesenchymal transition, autocrine mitogen production, immunosuppression and migration. Therefore, paradoxically, TGFβ can function as either a tumour suppressor or tumour promoter depending on cellular context and understanding of the molecular basis of this switch is crucial to deciphering the therapeutic potential of the pathway (Massagué, 2008b; Ikushima and Miyazono, 2010b; Inman, 2011a; Wakefield and Hill, 2013; Colak and Ten Dijke, 2017).

Inactivation of core components of the TGFβ signalling pathway, either by mutation or allelic loss of heterozygosity (LOH), can enable cancer cells to evade the tumour suppressive effects of TGFβ. Thus far, the most frequently mutated components of the pathway are the TGFβ type I and type II receptors and the common mediator SMAD4, all of which have been associated with the pathogenesis of multiple different human cancers (Levy and Hill, 2006). SMAD4 (originally termed deletion target in pancreatic carcinoma, locus 4; DPC4) was initially identified as a candidate tumour suppressor gene in human pancreatic carcinomas (Hahn *et al.*, 1996). Allelic loss at human chromosome 18q occurs in approximately 90 percent of human pancreatic carcinomas and over 60 percent of colorectal carcinomas (Padua and Massagué, 2009). The *SMAD4/DPC4* gene, along with another candidate tumour suppressor gene DCC, was found to be within the minimally lost region on chromosome 18q21 (Thiagalingam *et al.*, 1996) and homozygous deletions of the *SMAD4/DPC4* gene were observed in approximately 30 percent of pancreatic carcinomas analysed (Hahn *et al.*, 1996).

Furthermore, mutations in SMAD4 that result in protein instability have been identified that are associated with the pathogenesis of acute myelogenous leukaemia and pancreatic adenocarcinoma (Wan *et al.*, 2005; L. Yang *et al.*, 2006). A missense mutation in the SMAD4 MH1 domain (c.305C>T, Pro102Leu) and a frameshift mutation that results in premature termination within the MH2 domain (Δ483-552) undergo rapid proteasomal degradation via Skp1, Cullin, F-box containing complex (SCF)-β-TrCP E3 ubiquitin protein ligase-mediated polyubiquitylation (L. Yang *et al.*, 2006).

Evasion of TGFβ-mediated tumour suppression can also occur as a result of defects in downstream signalling responses. As alluded to previously, TGFβ-induced cytostasis is one of the critical tumour suppressive responses, however epigenetic silencing of the TGFβ target genes $p15^{INK4B}$ and $p21^{CIP1}$ by aberrant DNA methylation and histone modifications have been implicated in the pathogenesis of acute myelogenous leukaemia (Geyer, 2010). In addition, homozygous deletion of the *$p15^{INK4B}$* gene has been reported in human glioblastoma multiforme (GBM) (Jen *et al.*, 1994) and the tumour suppressive function of $p15^{INK4B}$ has also been demonstrated in murine models (Krimpenfort *et al.*, 2007). The *$p15^{INK4B}$* gene (*CDKN2B*) is located on human chromosome 9p21, within the same locus as two other cyclin-dependent kinases inhibitors (CDKIs) *$p16^{INK4A}$* (*CDKN2A*) and *$p14^{ARF}$* (encoded by an alternative reading frame of *CDKN2A*). Homozygous deletion of both *CDKN2B* and *CDKN2A* conferred a higher tumour predisposition and the development of a wider range of tumours compared with *CDKN2A* deficient mice, with the loss of $p15^{INK4B}$ specifically promoting the development of skin squamous and basal cell carcinomas and various soft tissue sarcomas (Krimpenfort *et al.*, 2007). Thus, defective cytostatic gene responses resulting from either epigenetic inactivation or allelic deletion can result in the abrogation of TGFβ tumour suppression (Massagué, 2008a).

Cancer cells which successfully evade the tumour suppressive effects of TGFβ signalling can subsequently co-opt the pathway and switch TGFβ signalling to a tumour promoting state. One of the mechanisms by which this occurs is through the production of autocrine mitogenic factors which can promote cellular proliferation (Massagué, 2008b). *In vitro* studies have demonstrated that hyper-diploid GBM cells proliferate in response to TGFβ stimulation, which is mediated via the induction of platelet-derived growth factor subunit B (PDGFB, also referred to as PDGF-2) (Jennings and Pietenpol, 1998). However, the ability of TGFβ to induce the expression of PDGFB in glioblastoma cells is dependent on the methylation

state of the *PDGFB* gene promoter (Bruna *et al.*, 2007). Therefore, epigenetic regulation of specific target genes can determine whether TGFβ exerts tumour suppressive or tumour promoting functions depending on cancer cell context (Kesari, Jackson-Grusby and Stiles, 2007).

The ability to induce and sustain angiogenesis (*i.e.* the growth of nascent blood vessels) is considered to be a fundamental acquired capability of developing tumours and is thus referred to a 'hallmark of cancer.' The importance of angiogenesis in the context of tumorigenesis can be exemplified by the observation that tumours developing in transgenic mice are susceptible to angiogenic inhibitors (Hanahan and Weinberg, 2000). TGFβ has been to shown to stimulate angiogenesis through the induction of the angiogenic factors vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF), and the TGFβ-mediated induction of CTGF expression has been reported to contribute to breast cancer metastasis (Sánchez-Elsner *et al.*, 2001; Kang *et al.*, 2003).

1.4.2 TGFβ-induced fibrosis

Fibrosis is a pathophysiological condition predominantly characterised by the excessive and aberrant deposition of extracellular matrix (ECM) components including collagen, fibronectin, laminin and vitronectin as a consequence of chronic myofibroblast activation (Krieg, Abraham and Lafyatis, 2007; Gordon and Blobe, 2008). Under physiological conditions, synthesis and deposition of ECM components occurs as part of the normal wound healing response, however tissue fibrosis is generally considered to be a failure of this response to terminate (Leask and Abraham, 2004). The pathological accumulation of ECM components can occur in multiple different tissues where it can interfere with normal organ function and unabated can result in organ failure (Gordon and Blobe, 2008).

Research has demonstrated that TGFβ is a critical regulator of ECM production and it is therefore unsurprising that aberrant TGFβ signalling has been implicated in the pathogenesis of tissue fibrosis. TGFβ has been shown to directly upregulate the transcription of both collagen and fibronectin, principal components of the ECM (Ignotz and Massagué, 1986). Moreover, the TGFβ-mediated transactivation of the *COL1A2* gene (collagen alpha-2(I) chain, also referred to as type I collagen) has been shown to be dependent on SMAD3 in human dermal fibroblasts (Chen *et al.*, 1999; Verrecchia, Chu and Mauviel, 2001).

Furthermore, the importance of SMAD3 in mediating TGFβ-induced fibrosis is exemplified by a number of observations including that SMAD3 deficient mice exposed to ionising radiation exhibit decreased radiation-induced fibrosis, with reduced myofibroblast recruitment, compared with wild type mice (Flanders *et al.*, 2002). Additionally, SMAD3 deficient mice appear to be resistant to both bleomycin (Zhao *et al.*, 2002) and TGFβ-induced pulmonary fibrosis (Bonniaud *et al.*, 2004). In the bleomycin-induced pulmonary fibrosis model, SMAD3 deficient mice exhibited fewer fibrotic lesions and reduced hydroxyproline content in the lungs following bleomycin treatment. Furthermore, SMAD3 deficiency also resulted in reduced mRNA expression of the major ECM components type I pro-collagen and fibronectin in response to bleomycin. Although bleomycin treatment increased the mRNA expression of TGFβ1, there was no observable difference between wild type and SMAD3 deficient mice (Zhao *et al.*, 2002; Flanders, 2004).

In addition to directly regulating aspects of the wound healing response and hence the pathogenesis of tissue fibrosis, TGFβ can also indirectly promote these processes via the induction of profibrotic cytokines that can stimulate fibroblast proliferation. For example, connective tissue growth factor (CTGF) is a profibrotic cytokine, which following TGFβ-mediated transcriptional upregulation, can function in either an autocrine or paracrine manner to induce fibroblast proliferation (Kim, Sheppard and Chapman, 2018). One of the proposed mechanisms by which CTGF exerts is profibrotic effects is by enhancing the ability of TGFβ to bind to its cognate cell surface receptor kinases at low TGFβ concentrations (Abreu *et al.*, 2002; Leask and Abraham, 2004).

Furthermore, plasminogen activator inhibitor-1 (PAI-1, also referred to as serpin E1) is a well-defined transcriptional target of SMAD-dependent TGFβ signalling (Dennler *et al.*, 1998). PAI-1 is a member of the serine protease inhibitor (Serpin) superfamily and functions as the principal physiological inhibitor of the serine proteases tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Inhibition of t-PA and u-PA prevents the proteolytic cleavage of the zymogen plasminogen into its catalytically active form plasmin, itself a serine protease (Yasar Yildiz *et al.*, 2014). Plasmin is a critical mediator of fibrinolysis (*i.e.* the degradation of fibrin), a physiological process that prevents the development of thrombosis. However, plasmin, along with plasmin-dependent matrix metalloproteinases (MMPs), have important functions in the degradation of ECM protein components. Under pathological conditions, high levels of PAI-1 ultimately results in the

inactivation of plasmin and plasmin-dependent MMPs, contributing to the excessive accumulation of collagen and other ECM proteins that are characteristic of tissue fibrosis. Consequently, excessive levels of PAI-1 have been implicated in the pathogenesis of tissue fibrosis in multiple different organ types including hepatic, pulmonary, cardiac and renal (Ghosh and Vaughan, 2012).

In addition to upregulation of ECM components and profibrotic cytokines, TGFβ has also been proposed to mediate tissue fibrosis pathogenesis via the transcriptional repression of proteins involved in ECM degradation (Verrecchia and Mauviel, 2007). For example, TGFβ-activated SMAD3-SMAD4 complexes have been reported to abrogate the cytokine-induced expression of matrix metalloproteinase-1 (MMP-1) in dermal fibroblast cells (Yuan and Varga, 2001). MMP-1, alternatively referred to as interstitial collagenase, is involved in regulating ECM and has been reported to proteolytically cleave type I, II and III collagen (Matrisian, 1990). Thus, SMAD-mediated repression of MMP-1 gene transcription may be important for preventing excessive ECM degradation induced by inflammatory cytokines. However, aberrant TGFβ signalling may consequently promote the development of tissue fibrosis by downregulating enzymes required for ECM degradation.

1.4.3 Pharmacological intervention of TGFβ-associated human pathologies

Aberrant TGFβ signalling has been implicated in the pathogenesis of numerous human disorders including cancer, tissue fibrosis and heredity disorders (discussed in the preceding sections of this chapter) and as such has generated significant interest for drug development by pharmaceutical and biotechnology companies. However, as discussed previously, TGFβ signalling regulates a diverse array of cellular responses and therefore, the highly pleiotropic and context-dependent nature of pathway has provided a considerable challenge for pharmacological intervention (Connolly, Freimuth and Akhurst, 2012). Despite this, a number of potential therapeutics have been developed using a variety of different drug design strategies and have proceeded through preclinical assessment to clinical trials. These include anti-ligand antisense oligonucleotides, ligand-competitive peptides, monoclonal antibodies against ligands or receptors and small-molecule inhibitors (SMIs) targeting the serine-threonine kinase activity of the TGFβ receptors (Akhurst and Hata, 2012).

Antisense oligonucleotides (also referred to as antisense RNA; asRNA) have been under clinical development for a number of TGFβ-associated human pathologies. One example, Trabedersen (AP 12009), is an antisense oligodeoxynucleotide specific for human TGFβ2 mRNA that has progressed to clinical trials (Schlingensiepen *et al.*, 2006, 2011). Overexpression of TGFβ2 has been reported in numerous malignant tumours including glioblastoma, pancreatic carcinoma and melanoma. Therefore, targeting mRNA translation using antisense oligonucleotides to mediate downregulation of TGFβ2 protein synthesis is a potential therapeutic approach. However, although trabedersen exhibited efficacy and safety in phase II clinical trials for treatment of high-grade glioblastoma (Bogdahn *et al.*, 2011), there remains difficulties with systemic drug delivery of antisense oligonucleotides (Akhurst, 2017).

Along with antisense oligonucleotides, TGFβ ligands have also been targeted for clinical intervention using monoclonal antibodies. In therapeutic applications, monoclonal antibodies are advantageous due to their target specificity and extracellular mechanism of action, a discernible benefit when attempting to neutralise extracellular ligands. Although monoclonal antibodies are less convenient to administer intravenously, their inherent pharmacokinetic stability enables infrequent drug administration (Akhurst and Hata, 2012). A number of monoclonal antibodies have been developed that have proceeded through various stages of pre-clinical development however fresolimumab, a high-affinity fully humanised monoclonal antibody that neutralises the active forms of all three TGFβ isoforms, has progressed furthest in clinical development (Lonning, Mannick and McPherson, 2011; Rice *et al.*, 2015). In a phase I clinical study, fresolimumab was determined to be well tolerated in a cohort of patients with advanced malignant melanoma or renal cell carcinoma, with no dose-limiting toxicity observed (Morris *et al.*, 2014). Although this phase I study demonstrated preliminary evidence of anti-tumour efficacy that deserves further investigation, a subset of patients developed a variety of skin lesions including hyperkeratosis and reversible non-malignant keratoacanthomas (KA), with one patient also developing cutaneous squamous cell carcinoma (SCC) at the highest drug dosing level. Furthermore, a clinical study in patients with recurrent high-grade glioma demonstrated that fresolimumab was efficiently delivered to the tumour following intravenous administration (den Hollander *et al.*, 2015). Although no clinical benefits were observed in this study, it was nonetheless important as it was the first demonstration of blood-brain barrier (BBB) penetration and tumour uptake of a radiolabelled therapeutic monoclonal antibody in glioma patients.

A multitude of small-molecule inhibitors (SMIs) have been developed that specifically target the TGFβ type I receptor kinase and inhibit the phosphorylation and activation of SMAD2 and SMAD3. In general, these SMIs are ATP mimetics that competitively bind to the hydrophobic ATP-binding pocket within the kinase domain of the receptor (Yingling, Blanchard and Sawyer, 2004; Akhurst and Hata, 2012). A number of these type I receptor SMIs have been extensively utilised in TGFβ signalling research, including SB-505124, an inhibitor of the type I receptors ACVR1B (ALK4), TGFβR1 (ALK5) and ACVR1C (ALK7) (DaCosta Byfield *et al.*, 2004) that has been employed throughout this thesis.

Over the previous two decades, protein kinases have emerged as one of the most predominant drug targets in the field of oncology, with around 20 SMIs targeting protein kinases approved for clinical use (Cohen and Alessi, 2013). However, despite the prominent association between aberrant TGFβ signalling and cancer development and metastasis, only one SMI specifically targeting the TGFβ type I receptor, LY2157299 (developed by Eli Lilly and commercially referred to as galunisertib) has progressed to clinical trials to date. Although a number of trials have indicated a clinical benefit of galunisertib monotherapy in various different cancers (Akhurst, 2017), research has identified serious adverse effects associated with continuous administration of type I receptor SMIs. For example, pharmacological inhibition of TGFβ signalling using SB-525334, a pharmacological inhibitor of TGFβ type receptor, significantly decreased the incidence rate and tumour size of uterine leiomyomas (a tumour of mesenchymal origin) in Eker rats. However, in contrast, inhibition of TGFβ signalling by SB-525334 promoted the development of renal neoplastic lesions including adenomas and carcinomas in the same rat model (Laping *et al.*, 2007). Furthermore, it was observed in preclinical toxicity studies that administration of two different pharmacological inhibitors of the TGFβ type I receptor resulted in serious cardiac toxicity through the induction of heart valve lesions (Anderton *et al.*, 2011). Therefore, although pharmacological inhibition of TGFβ signalling appears to exhibit clinical benefit towards certain tumour types, it may also result in serious adverse effects and even promote the pathogenesis of other tumour types, which is perhaps unsurprising given the pleiotropic nature of TGFβ signalling and its ability to function as a tumour suppressor.

Although numerous human pathologies have been associated with aberrant TGFβ signalling, targeting the pathway for therapeutic intervention presents significant challenges. Due to the diverse array of physiological functions regulated by TGFβ signalling, complete

inhibition of the pathway using pharmacological approaches such as antisense oligonucleotides or monoclonal antibodies that target TGFβ ligands or small-molecule inhibitors of TGFβ receptor kinase activity may result in severe adverse effects in patients. For example, as discussed in section 1.5.1, TGFβ functions as a tumour suppressor in normal, premalignant cells and therefore any perturbation of this protective function may result in considerable detrimental outcomes in patients. Therefore, a comprehensive understanding of TGFβ signalling pathway regulation in different cellular contexts will be invaluable to the development of more safe and effective therapeutic strategies. Moreover, identification of novel regulatory components of the TGFβ pathway may reveal potential pharmacological targets that would enable the development of therapeutic interventions that specifically target discrete aspects of the pathway and reduce the likelihood of adverse side effects occurring.

1.5 AIMS OF THE PHD THESIS

The TGFβ signalling pathways perform critical functions during embryonic development and adult tissue homeostasis (section 1.4), and consequently malfunction of pathway components contributes the development of various human pathologies (section 1.5). The context-dependent nature of TGFβ signalling necessitates extensive pathway regulation and deciphering these ancillary regulatory components and contextual determinants may facilitate more targeted pharmacological approaches to treating TGFβ-related pathologies.

One of the most prevalent molecular mechanisms of regulating signal transduction pathways is through post-translational modification of signalling components. Extensive research has delineated multiple regulatory protein phosphorylation and ubiquitylation mechanisms throughout the TGFβ signalling pathway. Therefore, the inceptive broad aim of this doctoral project was to perform a pharmacological screen in an endogenous TGFβ-dependent transcriptional luciferase reporter cell line, in order to identify potential novel regulators of the TGFβ signalling pathway (section 3.1).

The data obtained from the pharmacological screen identified a number of small-molecule inhibitors which mediated a significant reduction in TGFβ-induced luciferase reporter activity. Due to time limitations and the available expertise and research tools within the MRC Protein Phosphorylation and Ubiquitylation Unit, I decided to focus on the most prominent hit that resulted from the screen, a small-molecule inhibitor of salt-inducible kinases (SIKs). Therefore, a subsequent aim of the project was to analyse the effect of multiple small-molecule kinase inhibitors that target SIKs on the TGFβ pathway and TGFβ-dependent transcriptional responses (sections 3.2 and 3.3 respectively).

In addition to employing the use of small-molecule kinase inhibitors, another aim of the project was to use alternative experimental methods by which to interrogate SIK function in order to further validate the initial observations (section 3.4). Manipulation of the upstream activating kinase of SIK isoforms, LKB1, or depletion of SIK protein levels provided further evidence for the role of SIKs in modulating TGFβ-dependent transcriptional responses. Moreover, genetic inactivation of SIK2 and SIK3 recapitulated the effect of SIK inhibition on TGFβ-mediated transcriptional induction of the target gene PAI-1, providing robust evidence for the role of SIKs in TGFβ signalling.

Finally, the fourth aim of the thesis project was to investigate the effect of SIK catalytic inhibition in the context of the TGFβ-regulated physiological processes such as cellular proliferation and apoptosis (section 3.6)

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Antibodies

For Western immunoblotting analysis, all primary IgG antibodies were used at 1:1000 dilution unless otherwise stated. Anti-Phospho-SMAD3 (S423/S425) Rabbit polyclonal IgG (600-401-919) was purchased from Rockland Inc. Anti-GFP Mouse monoclonal IgG (11814460001) was purchased from Roche. Anti-Phospho-SMAD2 (S465/S467) Rabbit polyclonal IgG (3101), anti-SMAD2/3 Rabbit monoclonal IgG (8685), anti-c-Myc Rabbit monoclonal IgG (5605), anti-$p27^{KIP1}$ Rabbit monoclonal IgG (3688), anti-$p21^{WAF1/CIP1}$ Rabbit monoclonal IgG (2947), anti-GAPDH Rabbit monoclonal IgG (used at 1:5000 dilution) (2118), anti-SIK2 Rabbit IgG (6919) were all purchased from Cell Signalling Technology (CST). Anti-PAI-1 Rabbit polyclonal IgG (ab66705), anti-CTGF Rabbit polyclonal IgG (ab6992) and anti-CRTC3 Rabbit monoclonal IgG (ab91654) were purchased from Abcam. Anti-Phospho-CRTC3 (S370) Sheep polyclonal IgG (S253D, 3rd bleed) and anti-SIK3 Sheep polyclonal IgG (S373D, 3rd bleed) were generated by MRC PPU Reagents and Services. Species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies were used at 1:2500-5000 dilution. Rabbit anti-Sheep polyclonal IgG (H+L) Secondary Antibody, HRP (31480) and Goat anti-Mouse polyclonal IgG (H+L) Secondary Antibody, HRP (31430) were purchased from Thermo Fisher Scientific. Goat anti-Rabbit polyclonal IgG (H+L), HRP-conjugated Secondary Antibody (7074) was purchased from CST.

2.1.2 Cytokines

Purified recombinant human TGFβ_1 was acquired from either R&D Systems or PeproTech and reconstituted in sterile 4 mM HCl containing 1 mg mL^{-1} bovine serum albumin (BSA) (Sigma-Aldrich). Purified recombinant human BMP-2 was obtained from R&D Systems and reconstituted in sterile 4 mM HCl containing 0.1% (w/v) BSA. Aliquots of both cytokines were prepared at 1000-fold the required concentration and stored at -20°C.

2.1.3 Small-molecule pharmacological inhibitors

The small-molecule inhibitors used in this thesis are detailed in the table below. All the inhibitors were reconstituted at 10 mM in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and aliquots stored at -20°C. The inhibitors were used at the concentrations and durations indicated in the respective figure legends. For all experiments involving the use of inhibitors, an equivalent volume of DMSO was used as a control.

Small-molecule inhibitor	Company	Product number
Bosutinib	Cayman Chemical	12030
Dasatinib	Cayman Chemical	11498
HG-9-91-01	MRC-PPU Reagents and Services	-
LDN193189	MRC-PPU Reagents and Services	-
MRT199665	MRC-PPU Reagents and Services	-
MRT67307	Sigma-Aldrich	SML0702
SB-505124	Sigma-Aldrich	S4696

Table 1. Details of the small-molecule inhibitors employed in this thesis including the respective company and product number information

2.1.4 RT-qPCR primers

All the oligonucleotide PCR primers utilised in this thesis were purchased from Invitrogen and resuspended at 100 mM in Nuclease-free H_2O and subsequently stored at -20°C. The primer sequences were either designed using the National Centre for Biotechnology Information (NCBI) Primer-BLAST online software tool (Ye *et al.*, 2012) or obtained from peer-reviewed published literature.

Target gene	Oligonucleotide sequence (5’ – 3’)
CTGF (forward)	GGA GAT TTT GGG AGT ACG G
CTGF (reverse)	TAC CAA TGA CAA CGC CTC CT

GAPDH (forward)	TGC ACC ACC AAC TGC TTA GC
GAPDH (reverse)	GGC ATG GAC TGT GGT CAT GAG
PAI-1 (forward)	AGC TCC TTG TAC AGA TGC CG
PAI-1 (reverse)	ACA ACA GGA GGA GAA ACC CA
ID-1 (forward)	AGG CTG GAT GCA GTT AAG GG
ID-1 (reverse)	GAC GAT CGC ATC TTG TGT CG
SMAD7 (forward)	CTG TGC AAA GTG TTC AGG TG
SMAD7 (reverse)	TTG AGA AAA TCC ATC GGG TA
murine *18S rRNA* (forward)	GTA ACC CGT TGA ACC CCA TT
murine *18S rRNA* (reverse)	CCA TCC AAT CGG TAG TAG CG

Table 2. Details of the oligonucleotide primers and their associated sequence used for RT-qPCR experiments in this thesis project

2.1.5 Small interfering RNA (siRNA) oligonucleotides

All the siRNA oligonucleotides employed in this thesis were purchased from Dharmacon and are detailed along with their sequences in the table below. All siRNA oligonucleotides were resuspended at 20 µM in RNase-free 1x siRNA Buffer (Dharmacon) according to the manufacturer's protocol and subsequently stored at -20°C.

siRNA	Oligonucleotide sequence	Product number
ON-TARGETplus SMARTpool Human SIK1	ACGAUUAGAUUCAAGCAAU	J-003959-14
ON-TARGETplus SMARTpool Human SIK1	GGAGUACUGUCACGACCAU	J-003959-15
ON-TARGETplus SMARTpool Human SIK1	GAAAAUCUAUCGUGAGGUU	J-003959-16
ON-TARGETplus SMARTpool Human SIK1	GCUCGGACCUCAGUGGUUU	J-003959-17
ON-TARGETplus SMARTpool Human SIK2	GAAAAUCUACCGAGAAGUA	J-004778-11
ON-TARGETplus SMARTpool Human SIK2	AAUCAUGGCCGGUUAAAUG	J-004778-12
ON-TARGETplus SMARTpool Human SIK2	CAACAGGUCUCCAGUGAGC	J-004778-13
ON-TARGETplus SMARTpool Human SIK2	GAAAGCGUCUCCACUCUCC	J-004778-14
ON-TARGETplus SMARTpool Human SIK3	GAACAGCGACGAUGCUUAU	J-004779-08

ON-TARGETplus SMARTpool Human SIK3	AUAGGGAACUGCAUGGAUA	J-004779-09
ON-TARGETplus SMARTpool Human SIK3	GACAGUAAGAGUUCAAGUA	J-004779-10
ON-TARGETplus SMARTpool Human SIK3	CCACGCACCUCGUCACCAA	J-004779-11
ON-TARGETplus Control siRNA	UGGUUUACAUGUCGACUAAUU	-

Table 3. Details of the siRNA oligonucleotides and the associated sequence information used during this thesis project

2.1.6 Recombinant proteins

Recombinant proteins with either *N*-terminal GST or *N*-terminal maltose-binding protein (MBP) affinity tags were expressed and affinity purified by the protein production team within MRC PPU Reagents and Services at the University of Dundee. All the recombinant proteins used in this thesis are detailed in section 2.2.21 and are commercially available to researchers via the website (https://mrcppureagents.dundee.ac.uk).

2.2 METHODS

2.2.1 Mammalian cell culture

A-172 human glioblastoma, A549 human pulmonary adenocarcinoma, U2OS human osteosarcoma, HACAT human immortalised keratinocyte, HEK-293 human embryonic kidney and HeLa human cervical adenocarcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone), 2 mM *L*-glutamine (Gibco), 100 units mL^{-1} Penicillin (Gibco) and 100 µg mL^{-1} Streptomycin (Gibco) (hereafter referred to as D10F media). NMuMG murine mammary epithelial cells were cultured in D10F media supplemented with 10 µg mL^{-1} insulin (from bovine pancreas) (Sigma-Aldrich). Primary and immortalised mouse embryonic fibroblast (MEF) cells were cultured in DMEM supplemented with 20% (v/v) FBS, 2 mM *L*-glutamine, 100 units mL^{-1} Penicillin (Gibco), 100 µg mL^{-1} Streptomycin (Gibco), 1x Minimum Essential Medium (MEM) Non-Essential Amino Acids (NEAA) (Gibco) and 1 mM Sodium Pyruvate (Gibco). All cell lines were maintained at 37°C in a humidified atmosphere with 5% (v/v) CO_2 levels.

For subculture of cell lines, cells were washed once with sterile 1x Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco) and then incubated at 37°C with approximately 2 mL 1x 0.05% (w/v) Trypsin-EDTA (Gibco) to facilitate cell dissociation (duration dependent on the adherence of the cell line). Once dissociated, cells were subsequently resuspended in the appropriate culture media (detailed previously) to a final volume of 10 mL per 10 cm cell culture plate. Immediately prior to use, culture media and trypsin solution were pre-incubated to 37°C. All cell culture procedures were conducted in a laminar flow cabinet using aseptic technique and adhering to biological safety (category 2) regulations.

2.2.2 Cryopreservation of mammalian cell lines

Sub-confluent cells were washed once with 1x DPBS, dissociated from the culture plate using 1x 0.05% (w/v) Trypsin-EDTA (Gibco) and subsequently resuspended in culture media. The cell concentration and viability of the suspension was determined prior to centrifugation at 300 x g for 2 minutes. The culture media was subsequently aspirated, and the cell pellet resuspended in cryopreservation media (50% (v/v) culture media, 40% (v/v) FBS and 10% (v/v)

DMSO) at a concentration of approximately 1.0-2.0 x 10^6 cells mL^{-1} and equal volumes dispensed into 2.0 mL cryogenic vials. Cryogenic vials were then stored at -80°C for 24 hours in a cell-freezing container (Mr. Frosty Freezing Container; Thermo Fisher Scientific) to provide a controlled rate of freezing. The cryogenic vials were subsequently transferred to liquid nitrogen for long-term storage.

In order to thaw cells, cryogenic vials were incubated in a water bath at 37°C for approximately 2 minutes and cell suspension transferred into 10 mL of appropriate culture media. The cell suspension was centrifuged at 300 x g for 2 minutes and the resulting cell pellet resuspended in 10 mL culture media and subsequently transferred into a 10 cm cell culture plate.

2.2.3 Transformation of primary MEFs

Primary MEFs were transformed via retroviral-mediated transduction of the oncoprotein Simian virus 40 (SV-40) large T antigen. In order to produce the retroviral transduction media, HEK-293 FT cells were transfected with the following cDNA plasmids (amount/volume specified per 10 cm cell culture plate): SV-40 large T antigen (6.0 µg), Gag/Pol (3.8 µg) and vesicular stomatitis virus glycoprotein G (VSV-G) (2.2 µg) using Lipofectamine 2000 Transfection Reagent (Invitrogen) (36 µL). Transfection solution was prepared in 10 mL Opti-MEM Reduced Serum Media (Gibco) and incubated on cells for 6 hours at 37°C. Transfection media was subsequently aspirated and replaced with D10F media and incubated overnight (approximately 16 hours). Culture media was subsequently replaced with new D10F media and incubated for a further 24 hours. Viral transduction media was collected, filtered through 0.45 µm cellulose acetate membrane syringe filter and either used immediately or cryopreserved in liquid nitrogen prior to storage at -80°C. Polybrene (hexadimethrine bromide) (10 µg mL^{-1}) was added to the transduction media in order to enhance the retroviral transduction efficiency (Davis, Morgan and Yarmush, 2002) prior to incubation on the required cell line for 24 hours. Following transduction, cells were subjected to antibiotic selection using the appropriate culture media (detailed in section 2.2.1) supplemented with 200 µg mL^{-1} Hygromycin B Gold (InvivoGen).

2.2.4 Pharmacological screen

U2OS 2G transcriptional reporter cells (Rojas-Fernandez *et al.*, 2015) were seeded in 96-well cell culture plates (5.0 x 10^3 cells per well). Cells were subsequently incubated with a panel of 88 small-molecule inhibitors, in the presence or absence of TGFβ_1 stimulation (5 ng mL^{-1}) for 24 hours prior to lysis for subsequent luciferase assay (as detailed in section 2.2.14). The inhibitor screen was performed in triplicate and for each 96-well culture plate, the luminescence values were normalised to internal DMSO treated plate controls. Luminescence values from cells incubated with the inhibitors in the presence of TGFβ_1 were then normalised to the values obtained from cells incubated with the inhibitors alone to generate mean relative luciferase values for each condition. All the inhibitors in the panel were used at 1 µM concentration.

2.2.5 Treatment of cells with cytokines and small-molecule inhibitors

Prior to simulation with cytokines, cells were cultured in serum-free culture media for approximately 16 hours at 37°C in order to reduce basal cell signalling. In the case of NMuMG cells, cells were cultured in low-serum (1% (v/v) FBS) culture media (detailed in section 2.2.1) prior to cytokine stimulation. Cells were stimulated with 5 ng mL^{-1} TGFβ_1 or 6.25 ng mL^{-1} BMP-2 (unless otherwise stated) for the durations indicated in the respective figure legends. For all experiments involving cytokine stimulations, non-stimulated cells were included as negative controls.

The small-molecule inhibitors utilised in this thesis are detailed in section 2.1.4 and used at the concentrations and durations indicated in the respective figure legends. For all experiments involving the use of inhibitors, cells were treated with an equivalent volume of DMSO as a negative control.

2.2.6 Transfection of cells with siRNA

The siRNA oligonucleotides employed in this thesis are detailed in section 2.1.6. For all RNAi experiments, cells were seeded in 6-well cell culture plates (1.0 x 10^5 cells per well) unless

otherwise stated. Cells were transfected with 20 nM of siRNA oligonucleotide targeting the protein of interest or 20 nM non-targeting siRNA oligonucleotide as a negative control. Transfection solutions were prepared using 1 mL Opti-MEM Reduced Serum Media (Gibco) containing TransFectin Lipid Reagent (Bio-Rad) or Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) (4 µL per well) and the required volume of siRNA oligonucleotide. The transfection solution was subsequently vortexed for approximately 10 seconds and then incubated at ambient temperature for 15-20 minutes. For the purpose of siRNA transfections, the culture media on cells was exchanged for Opti-MEM Reduced Serum Media and 200 µL of the relevant transfection solution was added drop-wise per well. Approximately 6-8 hours following transfection, the transfection media was exchanged for the appropriate normal culture media and cells were stimulated with the required cytokines for the durations indicated in figure legends. Cells were lysed 72 hours post-transfection unless otherwise stated in the respective experiment figure legend.

2.2.7 Mammalian cell lysis

Unless otherwise stated, the lysis of mammalian cell cultures for protein extraction occurred as follows. The culture media was aspirated and cells were washed twice with cold 1x DPBS and incubated with lysis buffer (50 mM Tris/HCl pH 7.5, 270 mM sucrose, 150 mM sodium chloride, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (v/v) Nonidet P-40 (NP-40)) supplemented with Complete, EDTA-free Protease Inhibitors (Roche) (one tablet per 25 mL) for approximately 5 minutes on ice. The volume of lysis buffer used was dependent on the cell line, cell confluency and the size of the cell culture plate. Cell lysates were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 16,000 x g for 10 minutes at 4°C and either processed immediately or cryopreserved in liquid nitrogen prior to storage at -80°C. The protein concentrations of the cell lysate samples were determined using Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific) (further detailed in subsequent section 2.2.9). In order to denature and reduce the protein, cell lysate samples were subsequently diluted using 4x NuPAGE LDS (lithium dodecyl sulfate) sample buffer (Invitrogen) supplemented with 8% (v/v) 2-Mercaptoethanol (2-ME) (Sigma-Aldrich) and the

sample concentrations were equalised. Lysate samples were stored at either -20°C or -80°C and incubated at 95°C for 5 minutes immediately prior to SDS-PAGE analysis (section 2.2.12).

For experiments analysing apoptosis, the cell lysis protocol was amended to ensure that both adherent and non-adherent (*i.e.* apoptotic) cells were collected and occurred as follows. The culture media and PBS wash (both containing detached cells) were collected along with the trypsinised cells into 50 mL conical centrifuge tubes and centrifuged at 300 x g for 2 minutes. The supernatant was aspirated, and cell pellet resuspended in cold 1x DPBS followed by another centrifugation step as before. The cell pellet was subsequently lysed using an appropriate volume of lysis buffer and the samples prepared as described previously.

2.2.8 Protein concentration measurement

The protein concentration of cell lysate samples was determined using the Bradford assay method (Bradford, 1976) using the Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific). The Bradford assay is a colorimetric protein assay that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins under acidic conditions, which induces a shift in the absorbance maximum of the dye from 465 nm to 595 nm. In order to quantify the protein concentration of lysate samples, a standard curve was prepared using a dilution-series of bovine serum albumin (BSA) protein standard, with a concentration range from 0.0625 mg mL^{-1} to 1.0 mg mL^{-1}. To ensure the absorbance values of the lysate samples were within the range of the protein standard curve, the lysate samples were diluted in ultrapure H_2O (usually 10-fold dilution, however dependent on cell type and confluency) prior to performing the assay. The protein standards and diluted lysate samples were added to a 96-well microplate in triplicate (5 µL per well) along with the Bradford reagent (200 µL per well, equilibrated to ambient temperature prior to use) and incubated for approximately 5 minutes at ambient temperature. The absorbance values of all the sample were measured at 595 nm using a VersaMax microplate spectrophotometer and SoftMax Pro (version 4.8) software (Molecular Devices) and the protein concentrations of the lysate samples determined by reference to the protein standard curve. Baseline absorbance values were obtained using a blank solution (5 µL ultrapure H_2O, 200 µL Bradford reagent per well).

2.2.9 Endogenous CRTC3 immunoprecipitation (IP)

Cells were lysed for protein extraction and the protein concentration of lysate samples determined as detailed previously (section 2.2.7 and 2.2.8 respectively). 2 µg anti-CRTC3 polyclonal Sheep IgG (MRC PPU Reagents and Services, S277D, 2nd bleed) was added to 750 µg cell lysate and incubated for 1 hour at 4°C on a bench-top rotating platform. Lysate samples were subsequently added to 20 µL Protein G Agarose resin suspension (pre-washed three times with lysis buffer) and incubated for 1 hour at 4°C on a bench-top rotating platform. Lysate samples were subsequently centrifuged at 800 x g for 1 minute at 4°C and the resin washed three times with lysis buffer to remove unbound proteins. The agarose resin was subsequently resuspended in 60 µL 1x NuPAGE LDS sample buffer containing 2% (v/v) 2-mercaptoethanol and incubated at 95°C for 5 minutes to elute the immunoprecipitated proteins. Samples were then transferred to Corning Costar Spin-X centrifuge tube filters (0.22 µm cellulose acetate membrane) (Sigma-Aldrich) and centrifuged at 16,000 x g for 2 minutes to remove the agarose resin. The IP samples, along with total cell lysate and flow-through samples if required, were subsequently stored at -20°C prior to SDS-PAGE and Western immunoblot analysis (section 2.2.12 and 2.2.13 respectively).

2.2.10 Covalent conjugation of antibodies to protein G agarose

Protein G agarose resin and the required antibody (2 µg Ab per 10 µL agarose resin suspension) were incubated for 2 hours at 4°C with continuous rotation. The antibody-resin conjugate was subsequently washed three times with 100 mM sodium borate (pH 9.3). The antibody-resin was then resuspended in 100 mM sodium borate (pH 9.3) supplemented with 20 mM dimethyl pimelimidate (DMP) (5.2 mg mL-1) and incubated for 30 minutes at room temperature on a bench-top rotating platform (this incubation step was performed twice). Following the second incubation with DMP, the antibody-resin was washed four times with 50 mM glycine (pH 2.5) in order to remove any non-covalently conjugated antibody. The antibody-resin was subsequently washed twice with 200 mM Tris/HCl (pH 8.0) and then incubated in 200 mM Tris/HCl (pH 8.0) overnight at 4°C on a bench-top rotating platform. Following this, the covalently conjugated antibody-resin was resuspended in phosphate-

buffered saline (PBS) containing 0.01% (v/v) sodium azide (NaN_3) and subsequently stored at 4°C.

2.2.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Two different SDS-PAGE systems were employed in this thesis; self-cast SDS polyacrylamide gels using the ATTO Dual Mini Slab gel electrophoresis system (ATTO Corporation) or NuPAGE 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen) using either XCell SureLock Mini-Cell or XCell SureLock Midi-Cell systems (Invitrogen). The protein concentrations of the cell lysate samples were equalised using 1x NuPAGE LDS sample buffer containing 2% (v/v) 2-ME, incubated at 95°C for 5 minutes and briefly centrifuged prior to sample loading. Self-cast SDS polyacrylamide gels were prepared with an acrylamide concentration of either 10% or 12% and contained the following constituents; 375 mM Tris-HCl (pH 8.6), required volume of acrylamide/bis-acrylamide (29:1 molar ratio) solution (Sigma-Aldrich) and 0.1% (w/v) SDS. The polymerisation of acrylamide was catalysed via the addition of 0.075% (w/v) ammonium persulfate (APS) and 0.1% (v/v) tetramethylethylenediamine (TEMED). A small volume (approximately 600-800 µL) of 100% propan-2-ol was added to eliminate air bubbles and ensure the resolving gel formed a level surface. The resolving gel was incubated for at least 20 minutes at RT to ensure sufficient polymerisation and the propan-2-ol removed prior to the addition of the stacking gel. The stacking gel was prepared containing 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 4% (w/v) acrylamide, 0.075% (w/v) APS and 0.1% (v/v) TEMED and was added directly onto the polymerised resolving gel. A polypropylene sample comb (1 mm, 12-well) was immediately inserted and the gel allowed to polymerise for approximately 30 minutes at RT. Following polymerisation of the stacking gel, the sample comb was removed, and the sample wells rinsed with ultrapure H_2O. The SDS polyacrylamide gels were inserted into the ATTO Dual Mini Slab electrophoresis chamber, which was subsequently filled with 1x SDS-PAGE electrophoresis running buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 0.1% (v/v) SDS. For NuPAGE 4-12% Bis-Tris precast polyacrylamide gels, 1x MOPS (3-Morpholinopropane-1-sulfonic acid) SDS running buffer (Formedium) (final concentration 50 mM MOPS, 50 mM Tris Base, 3.47 mM SDS and 1 mM EDTA) was used. Reduced cell lysate samples (10-20 µg total protein unless stated otherwise in the respective figure legends) were

then loaded into the sample wells using sterile gel-loading micropipette tips. Precision Plus Protein All Blue Prestained Protein Standards (Bio-Rad), with molecular weights of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa, were combined with 1x NuPAGE LDS sample buffer containing 2% (v/v) 2-ME and used for molecular weight estimation. For the ATTO SDS-PAGE system, electrophoresis was conducted at constant voltage of 160 V for approximately 90 minutes. For the NuPAGE Bis-Tris SDS-PAGE system, electrophoresis was conducted at constant voltage of 180 V for approximately 60 minutes.

2.2.12 Western immunoblotting

Immediately following SDS-PAGE of cell lysate and IP samples, the resolved proteins were electrophoretically transferred onto Amersham Protran 0.45 µm nitrocellulose Western blotting membranes (GE Healthcare Life Sciences). Protein transfer was performed using either Mini Trans-Blot Cell (Bio-Rad) or Trans-Blot Cell (Bio-Rad) electroblotting system at 80 V (constant voltage) for 80 minutes at 4°C using 1x transfer buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% (v/v) methanol. The efficiency of protein transfer was assessed by reversible staining of the nitrocellulose membranes with Ponceau S (0.1% (w/v) in 5% acetic acid) solution (Sigma-Aldrich). In order to remove the Ponceau S staining prior to blocking, membranes were washed with Tris-buffered saline (TBS) (50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 0.1% (v/v) TWEEN 20 (Sigma-Aldrich), hereafter referred to as TBS-T (0.1%). Membranes were blocked using 5% (w/v) non-fat milk in TBS-T (0.1%) for 1 hour at RT on a bench-top platform rocker. The membranes were subsequently incubated with the appropriate primary antibodies (detailed in section 2.1.1) diluted in either 5% (w/v) milk-TBS-T (0.1%) or 4% (w/v) bovine serum albumin (BSA)-TBS-T (0.1%) overnight (approximately 16 hours) at 4°C with continuous agitation. Following this, membranes were washed three times for 5 minutes using TBS-T (0.1%) prior to incubation with the relevant species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were incubated with the HRP-conjugated secondary antibodies (detailed in section 2.1.1) diluted 1:2500-5000 in 5% (w/v) milk-TBS-T (0.1%) for 1 hour at RT on a bench-top platform rocker. The membranes were subsequently washed three times for 5 minutes using TBS-T (0.1%) prior to enhanced chemiluminescence detection. Membranes were incubated with Amersham

Enhanced Chemiluminescent (ECL) Western Blotting Detection Reagents (GE Healthcare Life Sciences) for approximately 2-4 minutes according to the manufacturer's instructions and subsequently exposed onto Medical X-Ray Film (Konica Minolta) or Amersham Hyperfilm ECL (GE Healthcare Life Sciences) under safelight conditions. The films were developed using an SRX-101A automated medical film processor (Konica Minolta).

2.2.13 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

The oligonucleotide PCR primers employed in this thesis are detailed in section 2.1.5. For all RT-qPCR experiments, cells were seeded in 6-well cell culture plates (1.0×10^5 cells per well) and cultured in serum-free or low-serum culture media prior to incubation with the required cytokines and/or inhibitors for the durations indicated in the respective figure legends. Total RNA was isolated from the cells using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol and the RNA concentration and quality subsequently determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesised from 0.5-1.0 µg of isolated RNA using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. All RT-qPCR reactions were conducted in triplicate and included 50% (v/v) SsoFast EvaGreen Supermix (Bio-Rad), 0.5 µM forward primer, 0.5 µM reverse primer and the required volume of cDNA. RT-qPCR experiments were performed using CFX96 or CFX384 Real-Time PCR Detection Systems (Bio-Rad). The Ct (cycle threshold) values for each gene of interest were normalised to the arithmetic mean Ct value of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S ribosomal RNA (rRNA) using Microsoft Excel software. The $2^{-\Delta\Delta Ct}$ relative quantification method was then used to analyse the relative changes in gene expression between control and treatment conditions (Livak and Schmittgen, 2001). GraphPad Prism software (version 8.0) was used to generate graphs and perform statistical analysis.

2.2.14 Luciferase transcriptional reporter assay

U2OS 2G transcriptional reporter cells (Rojas-Fernandez *et al.*, 2015) were seeded in 6-well cell culture plates (1.0×10^5 cells per well) and incubated with the required small-molecule

inhibitors/cytokines at the indicated concentrations and duration. Cells were subsequently washed twice with 1x DPBS at ambient temperature and lysed using 1x Cell Culture Lysis Reagent (CCLR; Promega) (280 µL per well, equilibrated to ambient temperature prior to use). Cell culture plates were incubated for approximately 5 minutes on bench-top platform rocker to ensure efficient cell lysis. Cell lysates were transferred to 1.5 mL microcentrifuge tubes and kept on ice. Lysate samples were vortexed for approximately 10 seconds, centrifuged at 12,000 x g for 2 minutes at 4°C and 200 µL of supernatant transferred to new 1.5 mL microcentrifuge tubes. Lysate samples were subsequently transferred to 96-well white flat-bottom cell culture microplate (Greiner Bio-One) (20 µL per well in triplicate). An equivalent volume of 2x Luciferase Assay Buffer (50 mM Tris/Phosphate pH 7.8, 16 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), 30% (w/v) glycerol, 1% (w/v) bovine serum albumin (BSA), 250 µM *D*-Luciferin, 8 µM sodium pyrophosphate) was subsequently added to each well and the microplate incubated for approximately 1 minute on a bench-top vibrating platform. Luminescence values were obtained using an EnVision 2104 Multimode Microplate Reader (PerkinElmer). The protein concentrations of each lysate sample were determined using Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific) as detailed in section 2.2.9 and used to normalise luminescence values.

2.2.15 Cellular fractionation (cytoplasmic/nuclear)

Extraction of separate cytoplasmic and nuclear protein fractions from cultured mammalian cells was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's protocol. The supplied lysis buffers (CER I and NER) were supplemented with 1x Complete, EDTA-free Protease Inhibitors (Roche) immediately prior to use. Subcellular fractions were reduced using NuPAGE 4x LDS sample buffer containing 8% (v/v) 2-mercaptoethanol and incubated at 95°C for 5 minutes prior to SDS-PAGE. Fractions were resolved by SDS-PAGE and analysed via Western immunoblotting as detailed previously (sections 2.2.12 and 2.2.13 respectively).

2.2.16 Annexin V staining assay

NMuMG murine mammary epithelial cells were incubated with SB-505124 (1 μM), MRT199665 (1 μM) or an equivalent volume of DMSO, in the presence or absence of recombinant human TGFβ$_1$ (5 ng mL^{-1}) for 12 hours. Following the cytokine/inhibitor treatment, both adherent and non-adherent (*i.e.* apoptotic) cells were collected into 50 mL conical centrifuge tubes, pelleted by centrifugation (300 x g, 2 minutes) and washed once using cold 1x DPBS. Cells were subsequently centrifuged (300 x g, 2 minutes), the cell pellets were resuspended in Annexin Binding Buffer, ABB (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and transferred to 1.5 mL microcentrifuge tubes. The required cell suspension samples were then incubated with Annexin V, Alexa Fluor 488 conjugate (Invitrogen; A13201) for 15 minutes at RT and protected from light. The appropriate samples were subsequently incubated with 5 μg mL^{-1} DAPI (4',6-diamidino-2-phenylindole) (Invitrogen). Samples were immediately analysed using a BD LSRFortessa Cell Analyser (BD Biosciences) and BD FACSDiva acquisition software (BD Biosciences). Annexin V Alexa Fluor 488 fluorescence was detected by excitation at 488 nm and emission at 530 $\pm$ 30 nm, and DAPI fluorescence was detected by excitation at 355 nm and emission at 450 $\pm$ 50 nm. Single cells were identified on the basis of forward light scatter (FSC) and side light scatter (SSC), and subsequently evaluated for Annexin V Alexa Fluor 488 and DAPI fluorescence. Data analysis was performed using FlowJo Single Cell Analysis Software (BD Biosciences). Generation of graphs and statistical analysis was performed using GraphPad Prism (version 8.0) software.

2.2.17 Crystal violet cellular viability assay

Protocol adapted from (Feoktistova, Geserick and Leverkus, 2016). NMuMG murine mammary epithelial cells were seeded in 96-well cell culture plates (1.0 x 10^4 cells per well) and incubated for 24 hours at 37°C to enable adherence of cells to culture plates. The inclusion of wells containing culture medium without cells were used as negative control wells. Following initial 24-hour incubation, culture media was aspirated and replaced with reduced serum (1% v/v FBS) DMEM (200 μL per well) supplemented with 10 μg mL^{-1} bovine insulin (Sigma-Aldrich) containing the required inhibitors or equivalent volume DMSO, with or without recombinant human TGFβ$_1$ (5 ng mL^{-1}) and incubated for a further 24 hours. Cells

were subsequently fixed using 10% (v/v) methanol/10% (v/v) acetic acid (100 µL per well) for 5 minutes at ambient temperature and subsequently washed with 1x PBS. Fixed cells were stained using 0.5% (w/v) crystal violet staining solution (0.5 g crystal violet powder (Sigma-Aldrich), 80 mL distilled H_2O and 20 mL methanol) (50 µL per well) for 20 minutes at ambient temperature on a bench-top platform rocker. Plates were subsequently washed carefully using tap water, inverted on filter paper to remove residual liquid and allowed to air-dry overnight. Following this, 200 µL methanol was added per well and incubated for 20 minutes at ambient temperature on a bench-top platform rocker. The absorbance value of each well was measured at 570 nm (OD_{570}) using a 96-well microplate spectrophotometer. The mean OD_{570} value of negative control wells (*i.e.* wells not containing cells) was subtracted from the values obtained from each well on the culture plate and the percentage of viable (*i.e.* attached) cells for each condition determined relative to the mean average OD_{570} value of non-stimulated (DMSO control) cells.

2.2.18 Cellular proliferation assay

The CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) was used to determine the relative number of viable HaCaT human immortalised keratinocyte cells in culture under certain conditions. HaCaT cells were seeded in 96-well cell culture plates (1.0 x 10^3 cells per well). Culture media (100 µL per well, refer to section 2.2.1), with or without recombinant human $TGF\beta_1$ (5 ng mL^{-1}), was exchanged every 24 hours for a duration of 7 days. Every 24 hours, one set of replicate wells were incubated with the CellTiter 96 AQueous One Solution Reagent (20 µL per well) for 2 hours at 37°C. The amount of soluble formazan product formed via the cellular reduction of the MTS tetrazolium compound is directly proportional to the number of metabolically active (*i.e.* viable) cells in culture. The absorbance of each well was measured at 490 nm (OD_{490}) using a 96-well microplate spectrophotometer and the absorbance values for each well were normalised to the OD_{490} values from wells containing culture media but without cells.

2.2.19 Removal of GST affinity tag from purified recombinant proteins

The glutathione S-transferase (GST) affinity tag of purified recombinant proteins was removed via proteolytic cleavage using PreScission Protease. PreScission Protease is a fusion protein of human rhinovirus (HRV) 3C protease and GST (Walker *et al.*, 1994) which specifically cleaves between the Gln and Gly residues of the amino acid recognition sequence Leu-Glu-Val-Leu-Phe-Gln/Gly-Pro (LEVLFQ/GP) (Cordingley *et al.*, 1990). The PreScission Protease was added to the GST-tagged recombinant protein at a ratio of 1:100 by weight and incubated overnight (approximately 16 hours) at 4°C with continuous rotation. Efficient cleavage of the GST tag from the recombinant protein was analysed via SDS-PAGE and subsequent Coomassie protein staining.

2.2.20 *In vitro* protein kinase assay

21 µL reaction solutions were prepared containing 200 ng of protein kinase and 2 µg of substrate protein in 1x kinase assay buffer (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 10 mM magnesium acetate, 0.1% (v/v) 2-mercaptoethanol and 0.1 mM [γ^{32}P]-ATP). Reactions were conducted at 30°C for 30 minutes at 1050 rpm and terminated via the addition of 7 µL NuPAGE 4x LDS sample buffer containing 8% (v/v) 2-mercaptoethanol. For *in vitro* kinase assays involving the use of small-molecule inhibitors, reaction solutions containing all the required components were incubated at 30°C for 10 minutes at 1050 rpm prior to the addition of 0.1 mM [γ32P]-ATP. Reactions were then performed as detailed previously. Samples were incubated at 95°C for 5 minutes and subsequently centrifuged at 5.0 x 10^3 x g for 1 minute. Samples were loaded (20 µL per well) into NuPAGE 4-12% Bis-Tris precast polyacrylamide gel and resolved via SDS-PAGE (section 2.2.12). The polyacrylamide gel was subsequently stained with InstantBlue Coomassie Protein Stain (Expedeon) to visualise the resolved recombinant proteins and imaged using the ChemiDoc Imaging System (Bio-Rad). ^{32}P radioactivity was analysed via autoradiography using Amersham Hyperfilm (GE Healthcare Life Sciences).

DU number	Protein	Residues	Affinity tag	Expression
33547	TGFβRI^{T204D}	200-501	*N*-terminal GST	Baculovirus
19395	SMAD2	1-467	*N*-terminal GST	*Escherichia coli*
33974	SMAD3	1-425	*N*-terminal GST	*Escherichia coli*
19398	SMAD4	1-502	*N*-terminal GST	*Escherichia coli*
40321	SIK1	2-783	*N*-terminal MBP	Baculovirus
16624	SIK2	2-926	*N*-terminal GST	Baculovirus
16623	SIK3	2-1369	*N*-terminal GST	Baculovirus

Table 4. Details of the recombinant proteins used for *in vitro* protein kinase assays in this thesis

2.2.21 Statistical analysis

All experiments have a minimum of three biological replicates unless otherwise stated in the respective figure legend. In addition, all luciferase, RT-qPCR, cellular proliferation, annexin V staining and crystal violet staining experiments have at least three technical repeats for each biological replicate. The data are presented as the arithmetic mean with error bars denoting the standard error of the mean (SEM). The statistical significance of differences between experimental conditions were assessed using either Student's t-test or analysis of variance (ANOVA) with Bonferroni correction using GraphPad Prism (version 8.0) analysis software. Differences in the mean of experimental conditions was considered significant if the probability value (p-value) was <0.05. All immunoblotting figures are representative.

3 RESULTS

3.1 SMALL-MOLECULE INHIBITOR SCREEN IN ENDOGENOUS TGFβ-RESPONSIVE TRANSCRIPTIONAL REPORTER CELL LINE

3.1.1 Introduction

In order to identify novel regulatory components of the TGFβ signalling pathway, I employed a pharmacological approach using an endogenous transcriptional reporter cell line for the TGFβ pathway (Rojas-Fernandez *et al.*, 2015), previously generated through a collaboration between the Sapkota lab and the research group of Prof. Ron Hay (Centre for Gene Regulation and Expression, School of Life Sciences, University of Dundee).

The cell line was generated using the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) genome editing system to insert a polycistronic transcriptional reporter cassette immediately downstream and in-frame of the endogenous promoter region for the TGFβ-responsive target gene *plasminogen activator inhibitor 1* (*PAI-1*). The transcription of PAI-1 is potently induced in response to TGFβ stimulation in multiple different cell lines in a SMAD-dependent manner (Keeton *et al.*, 1991; Dennler *et al.*, 1998). Furthermore, the promoter region of the *PAI-1* gene has been frequently utilised in order to generate conventional luciferase-based bioassay reporter systems for the study of TGFβ-dependent transcriptional regulation (Abe *et al.*, 1994). Therefore, the PAI-1 promoter region represented an appropriate target for CRISPR-Cas9-mediated insertion of the transcriptional reporter cassette.

The CRISPR-Cas genome editing system involves the introduction of a DNA double-stranded break (DSB) at a targeted genomic locus mediated by the RNA-guided DNA endonuclease Cas9 (Jiang and Doudna, 2017). The specificity of the Cas9 nuclease is determined via a 20-nucleotide (nt) sequence within its associated single-guide RNA (sgRNA) which binds to the targeted genomic DNA sequence through Watson-Crick base pairing (Ran *et al.*, 2013). A U2OS human osteosarcoma cell line stably expressing the Cas9 nuclease was co-transfected with a vector encoding the sgRNA targeting the start codon region of the endogenous *PAI-1* gene together with a donor vector containing a dual-reporter cassette

flanked by two sequences (consisting of approximately 500 nucleotides) homologous to the *PAI-1* gene that facilitates homologous recombination (HR). Following HR, the reporter cassette was incorporated in-frame with the ATG start codon of the endogenous PAI-1 gene (*figure 3.1-A*). The transcriptional reporter cassette contained cDNA encoding the *firefly* luciferase enzyme, immediately followed by an internal ribosome entry site (IRES), green fluorescent protein (GFP) and the 2A self-cleaving peptide. The IRES element facilitates 5' cap-independent protein translation (Pelletier and Sonenberg, 1988), thereby enabling the separate expression of the luciferase enzyme and GFP. The 2A self-cleaving peptide ensures the separation of the GFP from the endogenous PAI-1 protein.

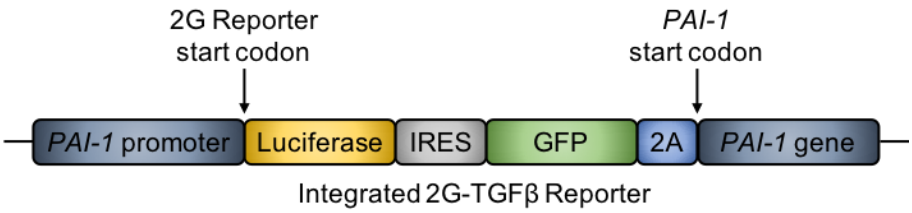


Figure 3.1A. Schematic representation of the endogenous TGFβ-responsive transcriptional reporter system
The dual-reporter cassette containing cDNA encoding both the firefly luciferase enzyme and GFP was inserted in-frame with the ATG start codon of the endogenous *PAI-1* gene in U2OS human osteosarcoma cells. The IRES element facilitates the separate expression of the luciferase enzyme and GFP, whilst the 2A self-cleaving peptide ensures the separation of the GFP protein from the endogenous PAI-1 protein.

The development of this endogenous transcriptional reporter system was termed second-generation (2G) TGFβ transcriptional reporter and therefore the cell line is hereafter referred to as U2OS 2G transcriptional reporter. This system presents a number of advantages over existing transcriptional reporter systems that are based upon the overexpression of exogenous cDNA expression vectors containing a promoter fragment or repeats of specific nucleotide sequences. Most significantly, our endogenous transcriptional reporter system takes into consideration the native chromatin context and gene regulatory components.

3.1.2 Characterisation of endogenous TGFβ transcriptional reporter cell line

For the purpose of this thesis, it was necessary to characterise the U2OS 2G TGFβ transcriptional reporter cell line prior to performing the small-molecule inhibitor screen. A time-course stimulation of wild type U2OS and U2OS 2G reporter cells with recombinant human TGFβ_1 over a period of 24 hours resulted in comparable levels of SMAD3 phosphorylation at serine 423 and serine 425 (the conserved carboxy-terminal residues subject to phosphorylation by the TGFβ type I receptor kinase), attaining maximal phosphorylation level at approximately 3 hours (figure 3.1B). As expected, expression levels of SMAD2 and SMAD3 proteins were comparable between wild type and 2G reporter cells and remained consistent over the stimulation time course (figure 3.1B). In the wild type U2OS cells, TGFβ_1 stimulation resulted in a concomitant increase in the protein expression of PAI-1, with the highest expression occurring at approximately 6 hours before decreasing back to basal levels. In contrast, PAI-1 protein expression is completely absent in the U2OS 2G reporter cells (figure 3.1B). In the initial study describing the development of the 2G transcriptional reporter cell line, sequence analysis of the *PAI-1* locus revealed that genomic integration of the dual-reporter cassette occurred in a heterozygous manner. Moreover, it was also revealed that the non-integrated *PAI-1* allele was disrupted via the insertion of a single nucleotide, resulting in a non-functional allele and consequently the absence of PAI-1 protein expression (Rojas-Fernandez *et al.*, 2015). However, TGFβ_1 stimulation of the U2OS 2G reporter cells induced a time-dependent increase in the expression of GFP, with the maximal expression observed at 24 hours (figure 3.1B). The differences in the protein expression dynamics of PAI-1 and GFP observed in the wild type and 2G reporter cells respectively may be explained by a number of factors. The decrease in PAI-1 protein expression observed after 6 hours of TGFβ stimulation may be partially explained by its physiological function as a major regulator of fibrinolysis (*i.e.* the enzymatic degradation of fibrin-containing blood clots). In order to perform this function, PAI-1 is secreted into the blood plasma by a number of different mammalian cells types including endothelial cells (Loskutoff *et al.*, 1983; Sawdey, Podor and Loskutoff, 1989), where it can subsequently bind and inhibit its principal substrates, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Chmielewska, Rånby and Wiman, 1983; Kruithof *et al.*,

1984). Consequently, the detection of PAI-1 protein expression via immunoblotting is likely to be influenced by its secretion into the culture media when culturing cells *in vitro*. Therefore, in all subsequent experiments involving either wild type U2OS or U2OS 2G transcriptional reporter cells, TGFβ stimulation was performed for 6 hours and 24 hours respectively (unless otherwise stated in figure legends).

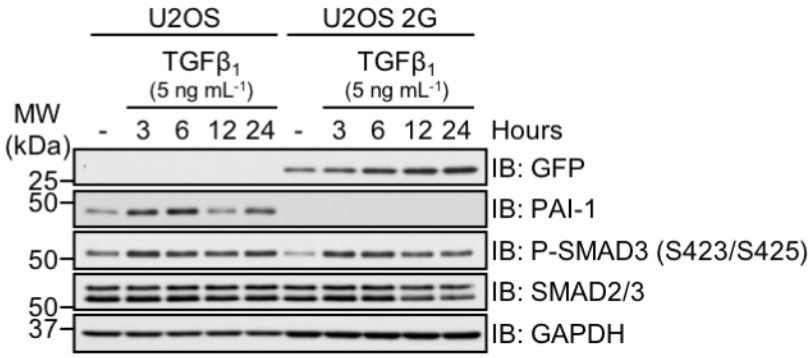


Figure 3.1B. Comparison of U2OS osteosarcoma wild-type and 2G transcriptional reporter cell lines.
U2OS wild type and 2G transcriptional reporter cells were stimulated with recombinant human TGFβ$_1$ (5 ng mL^{-1}) and lysed at the indicated time points. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. The immunoblot is representative of two independent experiments.

In order to further demonstrate that the 2G transcriptional reporter cells are responsive to TGFβ stimulation, experiments were performed involving the compound SB-505124, a small-molecule inhibitor of the TGFβ type I receptor kinases ACVR1B (ALK4), TGFβR1 (ALK5) and ACVR1C (ALK7) (DaCosta Byfield *et al.*, 2004). Stimulation of U2OS 2G transcriptional reporter cells with TGFβ resulted in a statistically significant 2.5-fold increase in luciferase activity compared with unstimulated control cells. However, this TGFβ-induced increase in luciferase activity was completely inhibited when cells were co-incubated with SB-505124 (*figure 3.1C-A*). Furthermore, using the same experimental conditions, it was

observed that SB-505124 inhibits TGFβ-mediated phosphorylation of SMAD3 and therefore completely inhibited the concomitant TGFβ-induced increase in GFP protein expression.

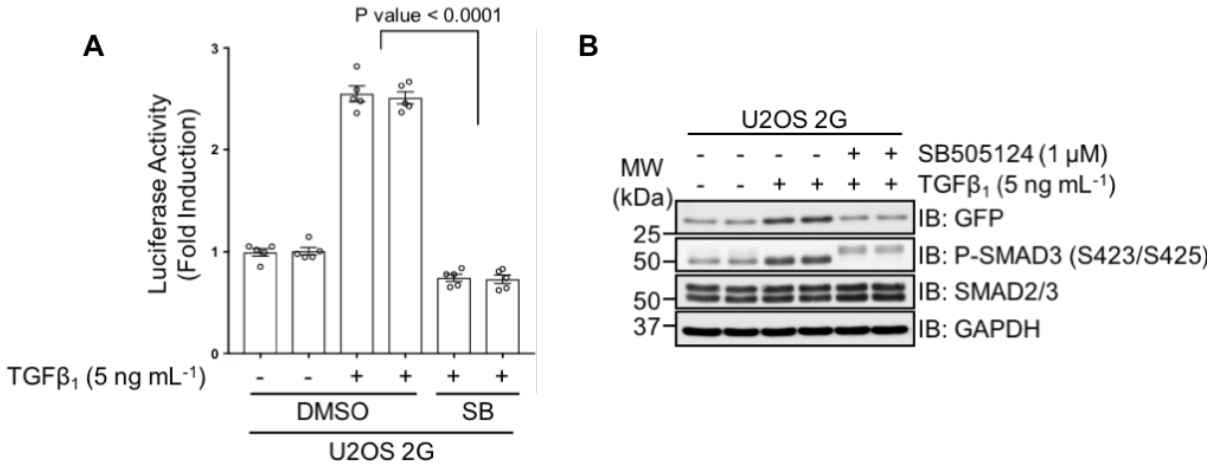


Figure 3.1C. The TGFβ type I receptor kinase inhibitor SB-505124 abrogates TGFβ-induced luciferase activity and GFP expression in 2G transcriptional reporter cells.
(A) U2OS 2G transcriptional reporter cells were stimulated with recombinant human TGFβ$_1$ (5 ng mL^{-1}) in the presence of SB-505124 (1 μM) or equivalent volume of DMSO, for 24 hours prior to cell lysis and luciferase assay. The experiment was performed five times and all luminescence values were normalised to the total protein concentration of the respective cell lysate sample. (B) U2OS 2G transcriptional reporter cells were stimulated with recombinant human TGFβ$_1$ (5 ng mL^{-1}) in the presence of SB-505124 (1 μM) or equivalent volume of DMSO, for 24 hours prior to cell lysis. Cell lysates (12 μg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. Immunoblots are representative of two independent experiments.

Therefore, collectively these experiments demonstrate that the U2OS 2G transcriptional reporter cell line is responsive to TGFβ stimulation and that the increase in luciferase activity or GFP expression is specifically dependent on TGFβ-mediated phosphorylation of SMAD3. Although slight differences were observed in protein expression dynamics between endogenous PAI-1 and GFP, the system effectively mimics the TGFβ-dependent transcriptional regulation of PAI-1 expression. I was therefore confident that this

transcriptional reporter cell line was a good system to employ in order to identify novel regulators of the SMAD-dependent signalling pathway.

3.1.3 Identification of putative hits obtained from the pharmacological screen

Once it had been confirmed that the U2OS 2G transcriptional reporter cells were a robust system for studying SMAD-dependent TGFβ-mediated transcriptional regulation, we employed them in a 96-well plate format pharmacological screen in order to identify potential novel regulators of the pathway. The panel used was composed of 88 small-molecule inhibitors predominantly targeting protein kinases but also a small number targeting enzymes of the ubiquitin system. The compounds were selected in order to ensure a broad coverage of the different kinase families within the human kinome. Furthermore, two compounds that are selective inhibitors of TGFβ type I receptor kinases were also included as positive controls; SB-505124 (DaCosta Byfield *et al.*, 2004) and A 83-01 (Tojo *et al.*, 2005). In addition, a number of internal DMSO plate controls were included for subsequent data normalisation.

The U2OS 2G transcriptional reporter cells were seeded in 96-well cell culture plates and incubated with the inhibitor panel either in the presence or absence of $TGF\beta_1$ stimulation (5 ng mL^{-1}) for a period of 24 hours (*figure 3.1-D*). All the inhibitors were used at 1 µM concentration. The cells were subsequently lysed, and the transcriptional reporter activity analysed using luciferase assays. As expected, the two selective and potent inhibitors of the type I receptor kinases that were employed as positive controls (SB-505124 and A 83-01) both resulted in statistically significant inhibition of TGFβ-induced luciferase activity compared with DMSO control treated cells (*figure 3.1-E*). Additionally, the compound D4476 also produced a statistically significant inhibition of TGFβ-induced luciferase activity. D4476 is a member of a compound series that was initially identified as inhibitors of TGFβRI (ALK5) (Callahan *et al.*, 2002). However, subsequent profiling of the compound against a panel of protein kinases revealed that it also inhibited the constitutively active serine-threonine protein kinase casein kinase 1 (CK1) with greater potency. D4476 was demonstrated to be an ATP-competitive inhibitor of CK1 with an IC_{50} value of 300 nM whereas it inhibited TGFβRI (ALK5) with an IC_{50} value of 500 nM (Rena *et al.*, 2004). D4476 is relatively specific for CK1 and

TGFβRI (ALK5) (MRC PPU Kinase Profiling Inhibitor Database; http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors) and is therefore commonly used as a CK1 inhibitor.

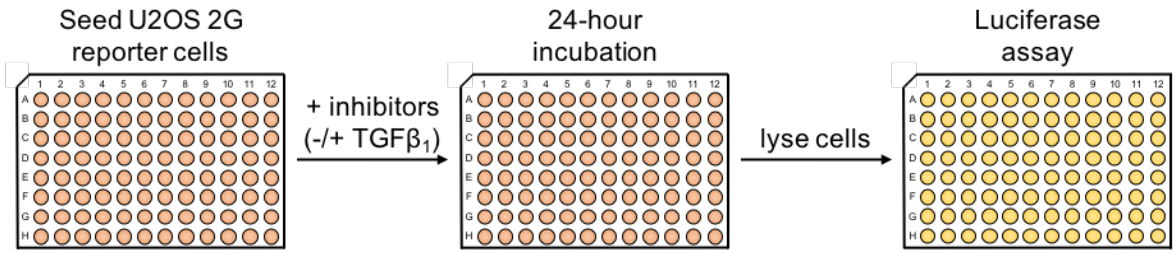


Figure 3.1D. Schematic representation of the experimental workflow for the small-molecule inhibitor screen in TGFβ-responsive 2G transcriptional reporter cells.
U2OS 2G transcriptional reporter cells were seeded in 96-well cell culture plates and then incubated with a panel of selective small-molecules inhibitors, in the presence or absence of $TGF\beta_1$ stimulation (5 ng mL^{-1}) for 24 hours. Cells were subsequently lysed and luciferase assays performed for each plate. The screen was performed in triplicate and the luminescence values for each plate were normalised to internal DMSO plate controls. The luminescence values for cells incubated with the inhibitor panel and TGFβ were then normalised to the values obtained for cells incubated with the inhibitors alone.

Plate number	Inhibitor	Molecular target	IC_{50} (nM)	Reference
1	A-83-01	ALK5	12	Tojo M. *et al* (2005)
2	MSC 2032964A	ASK1	93	Guo X. *et al* (2010)
3	KU-55933	ATM	13	Hickson I. *et al* (2004)
4	ETP-46464	ATR	25	Toledo L.I. *et al* (2011)
5	SU6668 (Orantinib)	Aurora B, Aurora C PDGFR, VEGFR, FGFR	35, 210	Sun L. *et al* (1999), Laird A.D. *et al* (2000), Godl K. *et al* (2005)
6	PLX-4720	$B\text{-}RAF^{V600E}$	13	Tsai J. *et al* (2008)
7	Imatinib	Abl	38	Buchdunger E. *et al* (1996)
8	Dasatinib	Abl, Src, Lck	0.5, 0.05, 0.4	Lombardo L.J. *et al* (2004)
9	LDN193189	ALK2, ALK3	5, 30	Yu P.B. et al (2008), Cuny G.D. *et al* (2008)
10	PCI-32765 (Ibrutinib)	BTK	0.5	Honigberg L.A. *et al* (2010)

11	PD-0332991 (Palbociclib)	CDK4, CDK6	11, 16	Toogood P.L., *et al* (2005)
12	CKI-7 dihydrochloride	CK1	6000	Chijiwa T. *et al* (1989)
13	D4476	CK1, ALK5	200, 500	Callahan J.F. *et al* (2002), Rena G. *et al* (2004)
14	GSK626616	DYRK3	0.7	Erickson-Miller C.L. et al (2007)
15	SB-505124	ALK4, ALK5	129, 47	DaCosta Byfield S. *et al* (2004)
16	AG-490	EGFR, JAK	2000, -	Gazit A. *et al* (1989), Gazit A. *et al* (1991), Meydan N. *et al* (1996), Wang L.H. *et al* (1999)
17	(Z)-4-hydroxytamoxifen	Estrogen receptor (ER)	-	Jordan V.C. *et al* (1977), Crewe H.K. *et al* (2002), Desta Z., *et al* (2004)
18	Zearalenone	Estrogen receptor (ER)		
19	CHIR 99021	GSK3		Ring D.B. (2003)
20	BI-605906	IKKβ	380	Clark K. (2011)
21	IRAK-4 kinase inhibitor A	IRAK4		Wang Z. (2006)
22	CP-690550 (Tofacitinib)	JAK1, JAK2, JAK3	112, 20, 1	Changelian P.S. (2003), Flanagan M.E. (2010)
23	INCB018424 (Ruxolitinib)	JAK1, JAK2	3.3, 2.8	Quintas-Cardama A. (2010)
24	JNK-IN-8	JNK1, JNK2, JNK3	5, 19, 1	Zhang T. (2012)
25	GSK2578215A	LRRK2	10	Reith A.D. *et al* (2012)
26	MAPKAP-K2 inhibitor A	MAPKAP-K2		Anderson D.R. *et al* (2007)
27	PF-3644022	MK2	5.2	Mourey R.J. *et al* (2010)
28	MRT199665	MARKs, SIKs		Clark K. *et al* (2012)
29	PD 0325901	MEK1, MEK2	0.3	Bain J. *et al* (2007), Barrett S.D. *et al* (2008)
30	ML-9 hydrochloride	MLCK	-	Nagatsu T. *et al* (1987)
31	CGP-57380	MNK1	2200	Knauf U. *et al* (2001)
32	CGP-57380 analog (SHN-093)	MNK	-	Buxade M. *et al* (2005)
33	AZD8055	mTORC1, mTORC2	0.8	Chresta C.M. *et al* (2010)
34	Amgen-NIK-28	NIK	-	Li K. *et al* (2013)
35	BIRB 796 (Doramapimod)	p38 MAPK $\alpha/\beta/\gamma/\delta$	38, 65, 200, 520	Pargellis C. *et al* (2002), Regan J. *et al* (2002), Kuma Y. *et al* (2005)
36	GSK2334470	PDK1	10	Najafov A. *et al* (2011)

37	GSK2606414	PERK	0.4	Axten J.M. *et al* (2012)
38	GDC-0941 (Pictilisib)	PI3K	3	Folkes A.J. *et al* (2008)
39	H-89 dihydrochloride	PKA	135	Davies S.P. *et al* (2000)
40	MK-2206	AKT1, AKT2, AKT3	5, 12, 65	Hirai H. *et al* (2010)
41	AX20017	PknG	390	Walburger A. *et al* (2004)
42	BI-2536	PLK1	0.8	Steegmaier M. *et al* (2007)
43	GSK461364	PLK1	2.2	Gilmartin A.G. *et al* (2009)
44	Necrostatin-1	RIP1		Degterev A. *et al* (2008)
45	Necrostatin-1 (inactive analog)	RIP1	-	Degterev A. *et al* (2008)
46	GSK429286	ROCK1	14	Goodman K.B. *et al* (2007)
47	BI-D1870	RSK1-4	31, 24, 18, 15	Sapkota G.P. *et al* (2007)
48	DMSO control	-	-	-
49	HG-9-91-01	SIK1, SIK2, SIK3	0.92, 6.6, 9.6	Clark K. *et al* (2012)
50	BML-258	SphK1	-	Paugh S.W. *et al* (2008)
51	SKI II	SphK1	500	French K.J. *et al* (2003)
52	VPS34-IN1	VPS34	25	Bago R. *et al* (2014)
53	STOCK1S-50699	WNK	37000	Mori T. *et al* (2013)
54	TC-S 7006	MAP3K8	50	Gavrin L.K. *et al* (2005)
55	Compound 401	DNA-PK	0.3	Griffin R.J. *et al* (2005)
56	DMSO control	-	-	-
57	MRT67307	IKKε, TBK1	160, 19	Clark K. *et al* (2011), Clark K. *et al* (2012)
58	HTH-01-015	NUAK1	100	Banerjee S. *et al* (2014)
59	IPA-3	PAK1	2500	Deacon S.W. *et al* (2008)
60	Rigel TRAF6 Inhibitor	TRAF6	-	-
61	Princeton's TrkA Inhibitor (compound 20h)	TrkA	0.6	Kim S.H. *et al* (2008)
62	AZ-23	TrkA, TrkB	2, 8	Thress K. *et al* (2009)
63	CGP-57380 analog (SHN-095)	MNK	-	Buxade M. *et al* (2005)
64	Mitoxantrone	USP11	3150	Burkhart R.A. *et al* (2013)
65	DMSO control	-	-	-
66	SJB3-019A	USP1	78	Mistry H. *et al* (2013)
67	DMSO control	-	-	-

68	Resveratrol	COX-1	-	-
69	MLN4924	NAE1	4.7	Soucy T.A. *et al* (2009)
70	BAY 11-7082	NF-κB	-	Pierce J.W. *et al* (1997)
71	-	-	-	-
72	-	-	-	-
73	ML240	p97 ATPase	100	Chou T.F. *et al* (2013)
74	ML241	p97 ATPase	100	Chou T.F. *et al* (2013)
75	Novartis compound 12a	PKD1	-	Meredith E.L. *et al* (2010)
76	MLN9708	20S proteasome	3.4	Kupperman E. *et al* (2010)
77	SRT 1720	SIRT1		Milne J.C. *et al* (2007)
78	Smac mimic (compound 3)	Smac	-	Li L. *et al* (2004)
79	DMSO control	-	-	-
80	VCP Inhibitor III	VCP ATPase	20	Magnaghi P. *et al* (2013), Polucci P. *et al* (2013)
81	Febuxostat	XO	1.2	Osada Y. *et al* (1993)
82	-	-	-	-
83	Blebbistatin	Myosin II ATPase	-	Limouze J. *et al* (2004)
84	Ischemin	p53 CBP	-	Borah J.C. *et al* (2011)
85	DBeQ	p97 ATPase		Chou T.F. *et al* (2011)
86	CCT007093	PPM1D	8400	Rayter S. *et al* (2008)
87	Harmaline	-	-	-
88	DMSO control	-	-	-
89	DMSO control	-	-	-
90	DMSO control	-	-	-
91	DMSO control	-	-	-
92	DMSO control	-	-	-
93	DMSO control	-	-	-
94	DMSO control	-	-	-
95	DMSO control	-	-	-
96	DMSO control	-	-	-

Table 5. The small-molecule inhibitors comprising the library used for pharmacological screening in TGFβ-responsive 2G transcriptional reporter cells

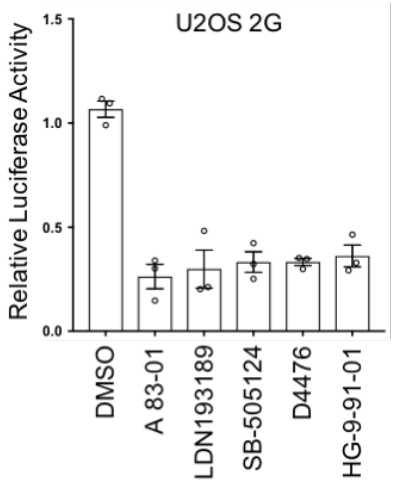
The above table provides information regarding the small-molecule inhibitor compounds used for the pharmacological screening conducted as part of this thesis project, and includes the intended molecular target, the *in vitro* IC_{50} (nM) if known and the relevant published research. The information was obtained from the online inhibitor database provided by the

MRC PPU International Centre for Kinase Profiling (http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors) and various commercial supplier websites.

Therefore, the inhibition of TGFβ-induced luciferase reporter activity observed with SB-505124, A 83-01 and D4476 provided a degree of confidence that the inhibitor screen was performed successfully, and that the data obtained was robust.

Furthermore, it was also observed that LDN193189, a selective inhibitor of the BMP type I receptor kinases ALK2, ALK3 and ALK6 (Cuny *et al.*, 2008), also resulted in the potent inhibition of TGFβ-induced luciferase activity. This most likely occurred because the concentration of LDN193189 used in the screen (1 μM) is 10-fold higher than typically used to inhibit the BMP type I receptors and therefore at a sufficiently high concentration to also inhibit the TGFβ type I receptors (Vogt, Traynor and Sapkota, 2011), although this observation was not subsequently verified experimentally in cells.

Unsurprisingly, the majority of the inhibitor compounds did not significantly impact TGFβ-induced transcriptional reporter activity. However, under these conditions it was discovered that HG-9-91-01, a potent inhibitor of salt-inducible kinase (SIK) isoforms, also significantly attenuated TGFβ-induced luciferase activity, suggesting that SIKs may be novel regulators of TGFβ-induced gene transcription. Therefore, I decided to focus the research on determining whether this initial observation was valid and the precise nature of any potential regulation of the TGFβ signalling pathway by SIK isoforms.



Mean Relative Luciferase Activity (± SEM)	Inhibitor	Target kinase
1.07 ± 0.04	DMSO	Control
0.26 ± 0.06	A 83-01	TGFβRI (ALK5)
0.30 ± 0.09	LDN193189	BMPRs (ALK2, ALK3)
0.33 ± 0.05	SB-505124	TGFβRI (ALK4, ALK5, ALK7)
0.33 ± 0.02	D4476	CK1, TGFβRI (ALK5)
0.36 ± 0.05	HG-9-91-01	SIKs

Figure 3.1E. Data obtained from the pharmacological screen using the TGFβ-responsive 2G transcriptional reporter cell line.
(A) Data obtained from the small-molecule inhibitor screen indicating the top 5 compounds which significantly suppressed the TGFβ-induced luciferase reporter activity compared to the DMSO control. The screen was performed in triplicate and the error bars represent the standard error of the mean (SEM). (B) Table indicating the mean relative luciferase activity values and the SEM values, in addition to the principal target protein kinases, of the top 5 compounds which suppressed TGFβ-induced luciferase reporter activity.

Mean Relative Luciferase Activity (± SEM)	Small-molecule inhibitor	Protein kinase target
1.07 ± 0.04	DMSO (mean average)	Control
0.26 ± 0.06	A 83-01	TGFβRI (ALK5)
0.30 ± 0.09	LDN193189	BMPRs (ALK2, ALK3)
0.33 ± 0.05	SB-505124	TGFβRI (ALK4, ALK5, ALK7)
0.33 ± 0.02	D4476	CK1, TGFβRI (ALK5)
0.36 ± 0.05	HG-9-91-01	SIKs
0.37 ± 0.13	GSK626616	DYRK3
0.39 ± 0.06	SJB3-019A	USP1
0.41 ± 0.07	ML240	p97 ATPase
0.46 ± 0.07	STOCK1S-50699	WNKs
0.47 ± 0.11	Amgen-NIK-28	NIK
0.53 ± 0.26	HTH-01-015	NUAK1
0.55 ± 0.06	CHIR 99021	GSK3
0.55 ± 0.05	SKI II	SphK1
0.57 ± 0.14	TC-S 7006	MAP3K8
0.58 ± 0.20	IRAK-4 kinase inhibitor A	IRAK4
0.58 ± 0.07	MLN9708	20S proteasome
0.60 ± 0.06	Tofacitinib	JAKs
0.60 ± 0.13	AG-490	EGFR, JAKs
0.62 ± 0.36	VCP Inhibitor III	VCP ATPase
0.63 ± 0.26	Orantinib	Aurora B/C, PDGFR, VEGFR
0.65 ± 0.07	ML-9 hydrochloride	MLCK
0.66 ± 0.03	Dasatinib	Abl, Src, Lck
0.69 ± 0.08	JNK-IN-8	JNKs
0.70 ± 0.15	ETP-46464	ATR
0.70 ± 0.12	MSC 2032964A	ASK1
0.71 ± 0.09	BI-605906	IKKβ
0.71 ± 0.09	MAPKAP-K2 inhibitor A	MAPKAP-K2
0.71 ± 0.13	MRT67307	IKKε, TBK1
0.71 ± 0.24	Ruxolitinib	JAK1, JAK2
0.71 ± 0.15	DMSO	Control
0.72 ± 0.22	CKI-7 dihydrochloride	CK1
0.74 ± 0.19	PLX-4720	B-RAF^{V600E}
0.74 ± 0.09	BIRB 796	p38 MAPK α/β/γ/δ
0.75 ± 0.20	DMSO	control
0.75 ± 0.07	PF-3644022	MK2
0.76 ± 0.24	SRT 1720	SIRT1
0.76 ± 0.12	(Z)-4-hydroxytamoxifen	Estrogen receptor (ER)
0.76 ± 0.03	Zearalenone	Estrogen receptor (ER)
0.78 ± 0.05	GSK429286	ROCK1
0.78 ± 0.07	MRT199665	MARKs, SIKs
0.78 ± 0.04	DMSO	Control
0.79 ± 0.22	AZD8055	mTORC1, mTORC2
0.81 ± 0.06	BML-258	SphK1
0.81 ± 0.21	Palbociclib	CDK4, CDK6
0.81 ± 0.20	H-89 dihydrochloride	PKA
0.82 ± 0.22	Compound 401	DNA-PK
0.82 ± 0.03	GSK2606414	PERK
0.82 ± 0.19	Smac mimic (compound 3)	Smac
0.83 ± 0.04	ML241	p97 ATPase
0.83 ± 0.05	Novartis compound 12a	PDK1
0.85 ± 0.09	Imatinib	Abl
0.85 ± 0.04	Harmaline	-
0.85 ± 0.21	DMSO	Control
0.85 ± 0.12	Febuxostat	XO
0.85 ± 0.11	Necrostatin-1 (inactive analog)	RIP1
0.86 ± 0.15	KU-55933	ATM
0.86 ± 0.17	PD 0325901	MEK1, MEK2
0.87 ± 0.11	Resveratrol	COX-1
0.87 ± 0.04	DMSO	Control
0.90 ± 0.07	CCT007093	PPM1D
0.90 ± 0.08	DMSO	Control
0.91 ± 0.02	Ischemin	p53 CBP
0.91 ± 0.07	DMSO	Control
0.91 ± 0.12	Necrostatin-1	RIP1
0.92 ± 0.10	-	-
0.93 ± 0.08	BAY 11-7082	NF-κB
0.93 ± 0.37	GDC-0941	PI3K
0.94 ± 0.09	GSK2334470	PDK1
0.94 ± 0.06	AX20017	PknG
0.94 ± 0.23	-	-
0.96 ± 0.17	MLN4924	NAE1
0.96 ± 0.06	DMSO	Control
0.98 ± 0.10	CGP-57380	MNK1
0.98 ± 0.25	DMSO	Control
0.98 ± 0.09	Princeton's TrkA inhibitor	TrkA
0.99 ± 0.23	MK-2206	AKT1, AKT2, AKT3
1.00 ± 0.06	GSK2578215A	LRRK2
1.01 ± 0.32	CGP-57380 analog	MNK1
1.02 ± 0.05	Ibrutinib	BTK
1.02 ± 0.11	GSK461364	PLK1
1.03 ± 0.08	DMSO	Control
1.06 ± 0.07	Blebbistatin	Myosin II ATPase
1.06 ± 0.02	BI-2536	PLK1
1.07 ± 0.01	VPS34-IN1	VPS34
1.07 ± 0.11	CGP-57380 analog (SHN-093)	MNK1
1.11 ± 0.21	DMSO	Control
1.12 ± 0.05	DMSO	Control
1.15 ± 0.03	DMSO	Control
1.18 ± 0.31	Rigel TRAF6 Inhibitor	TRAF6
1.26 ± 0.33	BI-D1870	RSK1-4
1.26 ± 0.11	DMSO	Control
1.30 ± 0.23	DMSO	Control
1.42 ± 1.04	DBeQ	p97 ATPase
1.97 ± 1.33	Mitoxantrone	USP11
2.64 ± 1.79	AZ-23	TrkA, TrkB
5.49 ± 2.69	IPA-3	PAK1

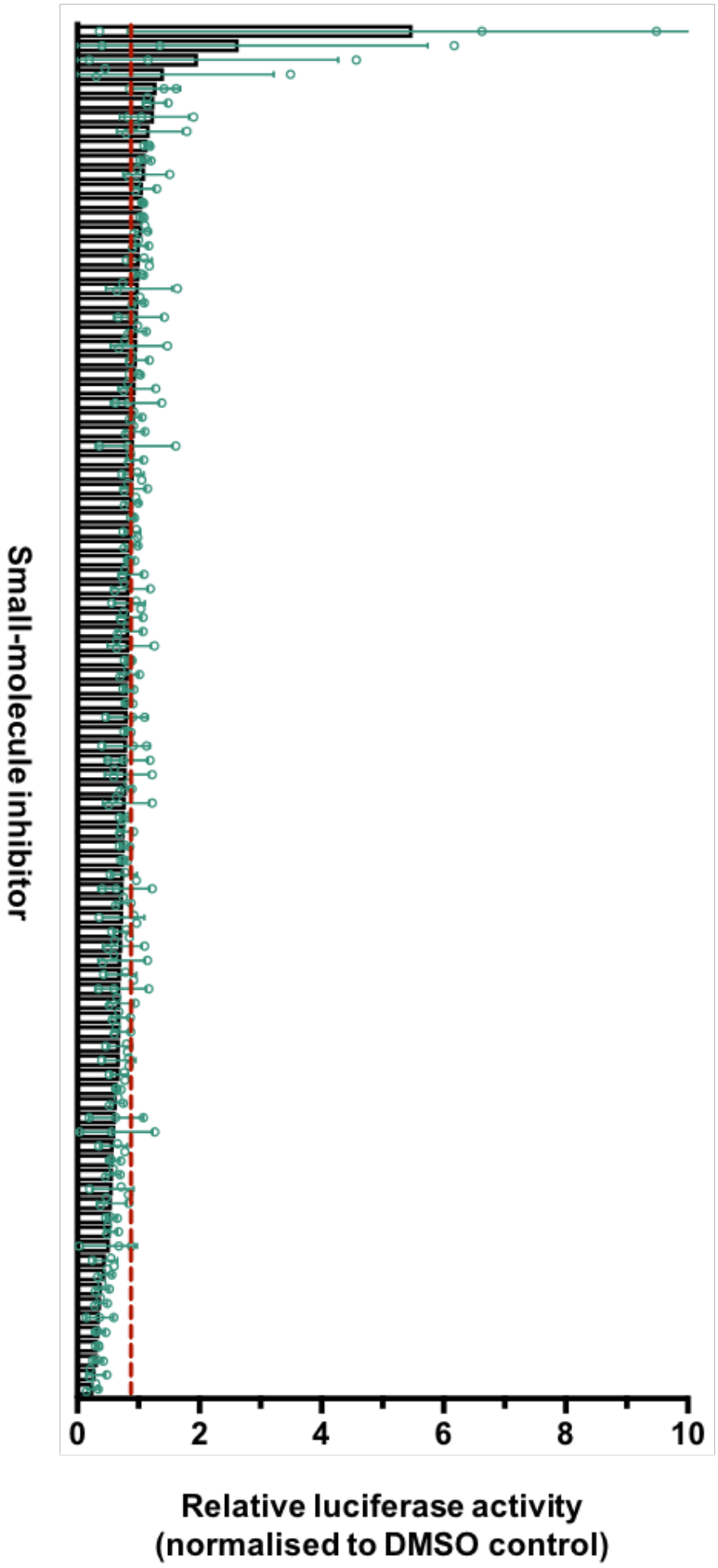


Figure 3.1F. Relative luciferase activity data from the pharmacological screen in TGFβ-responsive 2G transcriptional reporter cells
The luciferase reporter values for each small-molecule inhibitor compounds were normalised to internal DMSO plate controls to generate relative values in order to ascertain which compounds may inhibit or potentiate the TGFβ-dependent induction of the luciferase reporter enzyme. The values are the arithmetic mean obtained from three independent experiments and the error bars indicate the standard error of the mean (SEM). The red line denotes the overall average relative luciferase value obtained from the screen. Statistical analysis was performed using GraphPad Prism (version 8.0) software.

3.1.4 Discussion

The initial pharmacological screens performed as part of this doctoral project provided proof of concept that the endogenous 2G transcriptional reporter cells are a suitable system for conducting high-throughput screens. Although the experiments yielded a number of potential hits regarding novel regulators of the TGFβ, a number of difficulties and considerations arose that could be improved for any subsequent high-throughput screening (HTS) experiments. Firstly, the seeding of cells and subsequent addition of inhibitor compounds to the cells in a 96-well format was performed manually using multi-channel pipettes and therefore may be prone to pipetting errors. Therefore, the accuracy of these steps could be substantially improved by employing automated liquid dispensing equipment. Moreover, using automated liquid dispensing equipment would also enable larger screens (*e.g.* 384-well or 1536-well format) to be performed accurately and rapidly. Furthermore, automation of the screening procedure would have enabled the incorporation of multiple different concentrations of each compound instead of using a single concentration of 1 μM that was tested in our initial screens. This would have enabled us to obtain dose-response data for each inhibitor and therefore enhanced the robustness of the assay.

Although the transcriptional reporter system employed in this project is specific to TGFβ, in principal it would be possible to employ CRISPR-Cas genome editing technology to generate endogenous transcriptional reporter systems for any target gene of interest. This would provide a more relevant system for investigating the transcriptional regulation of specific genes or signalling pathways than systems which employ overexpression of exogenous cDNA reporter plasmids.

3.2 CHARACTERISATION OF SIK INHIBITORS IN THE CONTEXT OF TGFβ SIGNALLING

3.2.1 Introduction

Salt-inducible kinases (SIKs) are serine-threonine specific protein kinases that belong to the AMP-activated protein kinase (AMPK)-related subfamily. The AMPK-related subfamily consists of 12 members that are all closely related by sequence homology to the catalytic domain of AMPK and includes brain-specific serine-threonine protein kinase 1 and 2 (BRSK1 and BRSK2), microtubule affinity-regulating kinases 1-4 (MARKs1-4), maternal embryonic leucine-zipper kinase (MELK), NUAK1 and NUAK2 and three SIK isoforms (SIK1-3) (Bright, Thornton and Carling, 2009).

The catalytic subunits of AMPK (AMPKα1 and AMPKα2) were found to be direct substrates of the serine-threonine protein kinase LKB1 (also referred to as STK11) by three independent research groups (Hawley *et al.*, 2003; Hong *et al.*, 2003; Woods *et al.*, 2003; Shaw *et al.*, 2004). Activation of AMPK requires phosphorylation of a threonine residue (T172) within the activation loop of the catalytic subunit by LKB1 (Hawley *et al.*, 2003). Furthermore, interaction of LKB1 with its regulatory subunits, the pseudokinase STRADα/β (STE20-related kinase adaptor protein) (Baas *et al.*, 2003) and the scaffolding protein MO25α/β (also referred to as calcium-binding protein 39, CAB39) (Boudeau *et al.*, 2003), is required for complete activation of AMPK (Hawley *et al.*, 2003; Shackelford and Shaw, 2009).

LKB1 was initially identified as the tumour suppressor gene responsible for the development of the autosomal dominant inherited condition Peutz-Jeghers syndrome (PJS) which is primarily characterised by the presence of benign gastrointestinal polyps (Hemminki *et al.*, 1998). In addition, multiple epidemiological studies have reported an increased risk of developing gastrointestinal and non-gastrointestinal carcinomas including breast, ovarian and pulmonary in patients with PJS (Giardiello *et al.*, 1987; Boardman *et al.*, 1998; van Lier *et al.*, 2011; Riegert-Johnson, Westra and Roberts, 2012). Subsequent genetic analysis revealed that the LKB1 gene is frequently mutated in sporadic pulmonary adenocarcinomas, particularly non-small cell lung carcinomas (NSCLC) (Sanchez-Cespedes *et al.*, 2002; Ji *et al.*, 2007), and somatically mutated in at least 20 percent of cervical carcinomas (Wingo *et al.*,

2009), thereby demonstrating that LKB1 is an important tumour suppressor in multiple different cell types.

Following on from the identification of LKB1 as the upstream protein kinase involved in the activation of AMPK, LKB1 was also shown to phosphorylate and activate all the members of the AMPK-related subfamily (Lizcano *et al.*, 2004). As with the AMPK catalytic subunits, the LKB1-STRADα/β-MO25α/β complex catalyses the phosphorylation of a conserved threonine residue within the activation loop of the AMPK-related kinases, which enhances their catalytic activity more than 50-fold. An exception to this is MELK, whose catalytic activity was independent of LKB1 and is thus proposed to catalyse autophosphorylation of its own activation (T)-loop threonine residue.

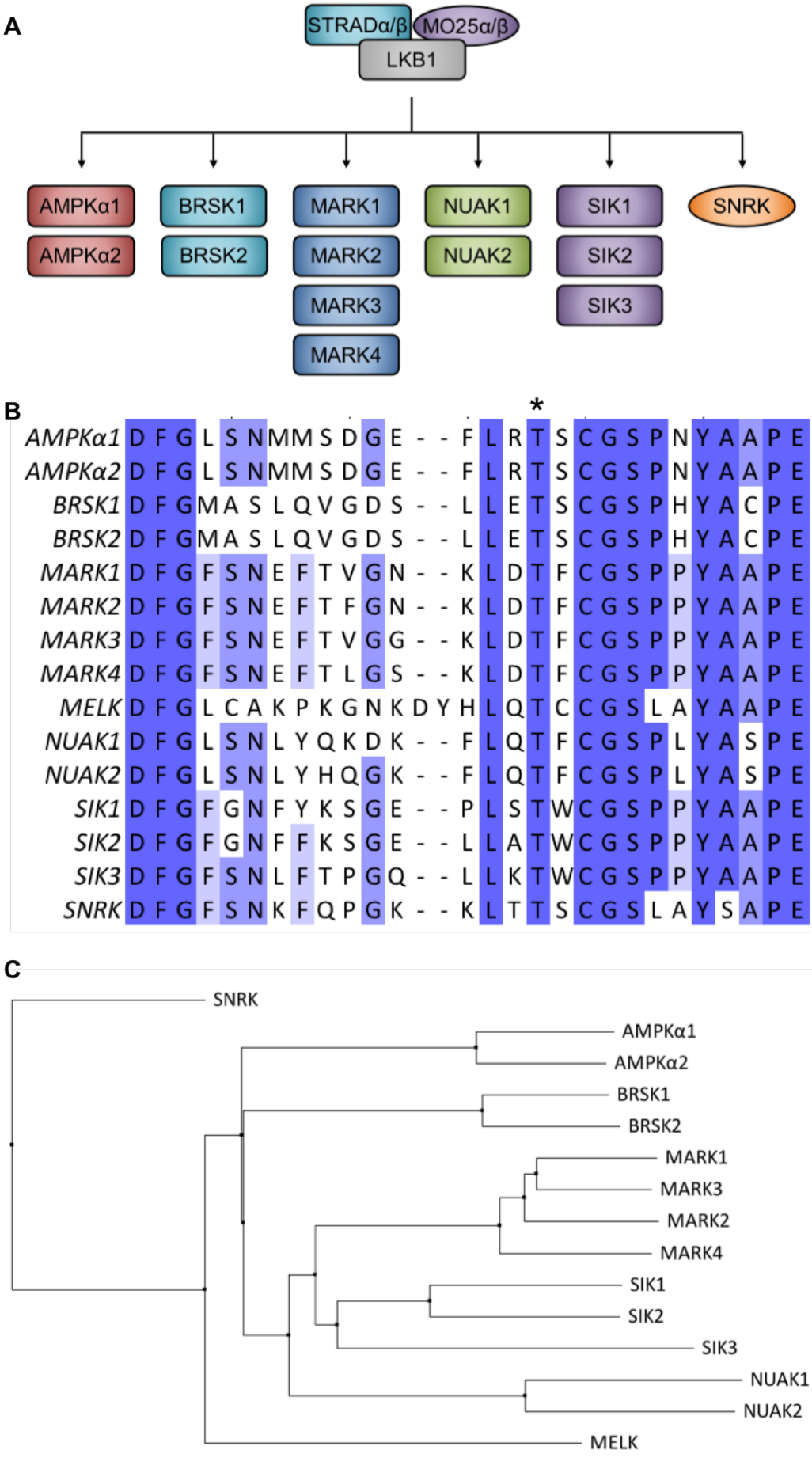

A
STRADα/β
MO25α/β
LKB1
AMPKα1
AMPKα2
BRSK1
BRSK2
MARK1
MARK2
MARK3
MARK4
NUAK1
NUAK2
SIK1
SIK2
SIK3
SNRK
B
*
AMPKα1 D F G L S N M M S D G E - - F L R T S C G S P N Y A A P E
AMPKα2 D F G L S N M M S D G E - - F L R T S C G S P N Y A A P E
BRSK1 D F G M A S L Q V G D S - - L L E T S C G S P H Y A C P E
BRSK2 D F G M A S L Q V G D S - - L L E T S C G S P H Y A C P E
MARK1 D F G F S N E F T V G N - - K L D T F C G S P P Y A A P E
MARK2 D F G F S N E F T F G N - - K L D T F C G S P P Y A A P E
MARK3 D F G F S N E F T V G G - - K L D T F C G S P P Y A A P E
MARK4 D F G F S N E F T L G S - - K L D T F C G S P P Y A A P E
MELK D F G L C A K P K G N K D Y H L Q T C C G S L A Y A A P E
NUAK1 D F G L S N L Y Q K D K - - F L Q T F C G S P L Y A S P E
NUAK2 D F G L S N L Y H Q G K - - F L Q T F C G S P L Y A S P E
SIK1 D F G F G N F Y K S G E - - P L S T W C G S P P Y A A P E
SIK2 D F G F G N F F K S G E - - L L A T W C G S P P Y A A P E
SIK3 D F G F S N L F T P G Q - - L L K T W C G S P P Y A A P E
SNRK D F G F S N K F Q P G K - - K L T T S C G S L A Y S A P E
C
SNRK
AMPKα1
AMPKα2
BRSK1
BRSK2
MARK1
MARK3
MARK2
MARK4
SIK1
SIK2
SIK3
NUAK1
NUAK2
MELK

Figure 3.2A. LKB1 is a master protein kinase that phosphorylates and activates AMPK and AMPK-related subfamily members.

(A) The tumour suppressor serine-threonine protein kinase LKB1, in complex with its regulatory subunits STRADα/β and MO25α/β, phosphorylates and activates the two AMPK catalytic subunits (AMPKα1 and AMPKα2) as well 12 other members of the AMPK-related family of serine-threonine protein kinases (*N.B.* the one exception is MELK, which is reported to undergo autophosphorylation in order to become catalytically active). **(B)** Sequence alignment of the activation segment of the AMPKα catalytic subunits and the 13 members of the AMPK-related family protein kinases. The activation segment is defined as the region between and including two conserved tripeptide motifs, the amino-terminal Asp-Phe-Gly (DFG) motif and the carboxy-terminal Ala-Pro-Glu (APE) motif. The DFG motif is situated within the magnesium-binding loop and participates in the coordination of an Mg^{2+} ion required for the catalytic activity. By contrast, although highly conserved in the activation segment of protein kinases, the function of the APE motif remains uncertain. LKB1 regulates the catalytic activity of AMPK and the majority of AMPK-related family members by phosphorylating a conserved threonine (Thr, T) residue within the activation (T)-loop (indicated by an asterisk), enhancing their activity over 50-fold. Multiple protein sequence alignment was performed in Jalview (version 2.10.5) software (Waterhouse *et al.*, 2009) using the Clustal Omega sequence alignment programme. Highly conserved amino acid residues are indicated by dark blue shading. **(C)** Phylogenetic tree derived from the protein sequence alignment of the catalytic protein kinase domain of AMPKα catalytic subunits and all the 13 members of the AMPK-related family. The phylogenetic tree was calculated using the neighbour-joining (NJ) method (Saitou and Nei, 1987) in Jalview (version 2.10.5). Abbreviations: AMPK, 5'-AMP-activated protein kinase; BRSK, Brain-specific serine/threonine-kinase; MARK, MAP/microtubule affinity-regulating kinase; MELK, Maternal embryonic leucine zipper kinase; NUAK, NUAK family SNF1-like kinase; SIK, Salt-inducible kinase; SNRK, Sucrose non-fermenting related kinase.

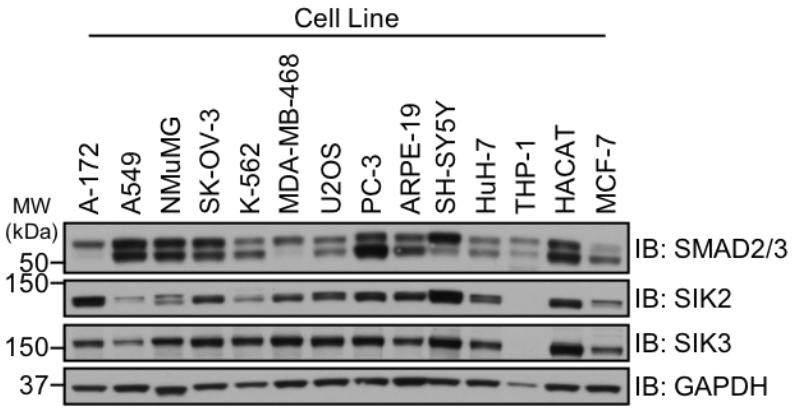


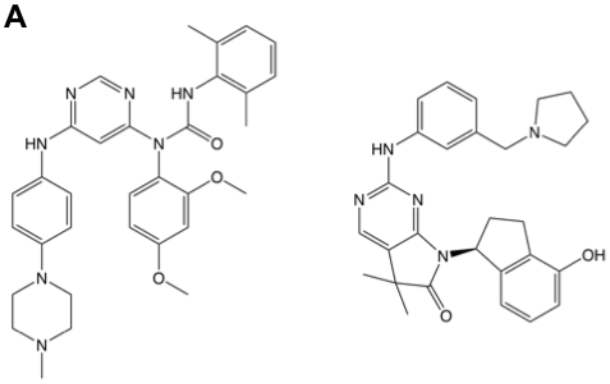
Figure 3.2B. Comparison of the protein expression of SIK2 and SIK3 isoforms across a panel of human and murine cell lines.
Cell lysate samples (12 µg total protein) obtained from multiple human and murine cell lines routinely used for *in vitro* cell culturing were resolved via SDS-PAGE and membranes subjected to immunoblotting with the indicated antibodies. Cell lines: A-172 (human glioblastoma), A549 (human pulmonary adenocarcinoma), NMuMG (murine mammary epithelial), SK-OV-3 (human ovarian adenocarcinoma), K-562 (human bone marrow-derived chronic myelogenous leukaemia (CML)), MDA-MB-468 (human mammary adenocarcinoma), U2OS (human osteosarcoma), PC-3 (human prostate adenocarcinoma), ARPE-19 (human retinal pigmented epithelium), SH-SY5Y (human bone marrow-derived neuroblastoma), HuH-7 (human hepatocellular carcinoma), THP-1 (human peripheral blood-derived acute monocytic leukaemia (AML-M5)), HACAT (human spontaneously transformed keratinocytes), MCF-7 (human mammary adenocarcinoma).

3.2.2 Two structurally distinct SIK inhibitors suppress TGFβ-induced luciferase transcriptional reporter activity

The pharmacological screen detailed previously identified SIK isoforms as potential novel regulators of TGFβ-induced gene transcription. Therefore, I sought to further validate this observation. In addition to HG-9-91-01, the compound MRT199665, a structurally distinct small-molecule inhibitor that is also known to inhibit SIK isoforms (Clark *et al.*, 2012) was also employed. Although both of these compounds are ATP-competitive kinase inhibitors, they exhibit contrasting specificities. MRT199665 is a potent inhibitor of most of the AMPK-related kinases with the exception of BRSK1 and BRSK2, and also inhibits the AMPK catalytic subunits (figure 3.2C). By contrast, HG-9-91-01 is a potent inhibitor of the three SIK isoforms however it does not inhibit AMPK or any of the other AMPK-related kinases (figure 3.2C). Therefore, it was examined whether both of these compounds could suppress TGFβ-dependent gene transcription. Indeed, both HG-9-91-01 and MRT199665 significantly attenuated TGFβ-

induced luciferase activity in the U2OS 2G transcriptional reporter cell line. This evidence provided greater confidence that the effect of these small-molecule inhibitors on TGFβ-mediated gene transcription was reproducible and not an experimental artefact

A



B

	IC_{50} values (nM)	
Protein Kinase	**HG-9-91-01**	**MRT199665**
LKB1	>10000	>10000
AMPKα1/α2	4500	10
BRSK1	>10000	>10000
BRSK2	8800	10000
MARK1	3300	2
MARK2	1600	2
MARK3	1900	3
MARK4	1300	2
MELK	2500	29
NUAK1	4280	3
NUAK2	145	120
SIK1	0.92	110
SIK2	6.6	12
SIK3	9.6	43

Figure 3.2C. The chemical structures and IC_{50} values of the pharmacological inhibitors HG-9-91-01 and MRT199665.

(A) The chemical structures of HG-9-91-01 and MRT199665, structurally unrelated small-molecule kinase inhibitors of SIK isoforms. The molecular structures were created using ChemDraw Professional software (version 17.0) (PerkinElmer). (B) IC_{50} (nanomolar) values for inhibition of LKB1, AMPK and the AMPK-related subfamily of protein kinases by HG-9-91-01 and MRT199665 in vitro. The information regarding chemical structures and IC_{50} values was obtained from (Clark *et al.*, 2012).

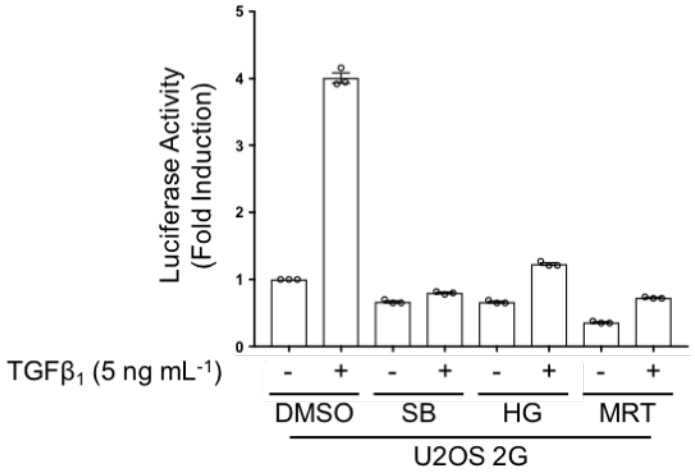


Figure 3.2D. The structurally distinct SIK inhibitors HG-9-91-01 and MRT199665 both attenuate TGFβ-induced luciferase reporter activity.
U2OS 2G transcriptional reporter cells were incubated with SB-505124 (1 μM), HG-9-91-01 (0.5 μM), MRT199665 (1 μM) or equivalent volume of DMSO in the presence or absence of TGFβ_1 stimulation (5 ng mL^{-1}) for 24 hours prior to lysis and luciferase assay. Luminescence values were normalised to the total protein concentration of the respective cell lysate sample and luciferase activity determined relative to unstimulated DMSO treated cells. The experiment was performed in triplicate and error bars represent standard error of the mean (SEM).

3.2.3 SIK inhibitors attenuate phosphorylation of CRTC3 in U2OS osteosarcoma cells

When employing the use of small-molecule inhibitors in research it is critical to ensure that the compounds are effectively inhibiting the intended molecular target at the concentration used and in the different cell types studied. In the context of pharmacological inhibitors of protein kinases, it is important to analyse the phosphorylation of known substrates of the target kinase. The SIK isoforms, along with MARKs and AMPK itself are known to catalyse the phosphorylation of cAMP-responsive element-binding protein (CREB)-regulated transcriptional co-activator (CRTC) isoforms (Altarejos and Montminy, 2011; Mair *et al.*, 2011; Sonntag *et al.*, 2017). In particular, research has demonstrated that SIK isoforms have an important function in the regulation of macrophage polarisation through phosphorylation of CRTC3. In macrophages, SIK isoforms have been shown to phosphorylate CRTC3 at serine 62, serine 162, serine 329 and serine 370, resulting in the interaction of CRTC3 with 14-3-3

binding proteins. The phosphorylation-dependent interaction between CRTC3 and 14-3-3 proteins inhibits the function of CRTC3 by preventing its translocation to the nucleus and thus supresses the transcriptional activity of CREB. Consequently, pharmacological inhibition of SIK isoforms promotes the dephosphorylation of CRTC3, thereby facilitating the translocation of CRTC3 to the nucleus where it can enhance CREB-dependent gene transcription, including IL-10 transcription, in response to Toll-like receptor (TLR) stimulation of macrophages (Clark *et al.*, 2012; MacKenzie *et al.*, 2013). Therefore, recent research has demonstrated that genetic or pharmacological inhibition of SIK isoforms can enhance the production of anti-inflammatory cytokines and thereby promote macrophages to adopt a regulatory phenotype (Sundberg *et al.*, 2014; Lombardi *et al.*, 2016a; Darling *et al.*, 2017). As a result, SIKs have been proposed as potential therapeutic targets for the development of novel anti-inflammatory drugs.

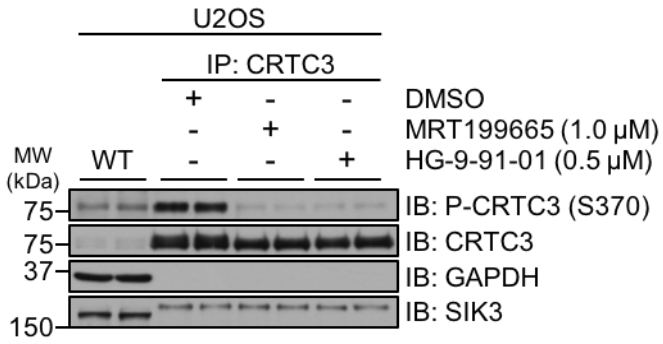


Figure 3.2E. The small-molecule inhibitors HG-9-91-01 and MRT199665 attenuate the phosphorylation of CRTC3 in U2OS osteosarcoma cells.
Wild type U2OS osteosarcoma cells were incubated with HG-9-91-01 (0.5 µM), MRT199665 (1 µM) or an equivalent volume of DMSO for 1 hour prior to cell lysis. Lysate samples (1 mg total protein) were subjected to immunoprecipitation of endogenous CRTC3 using anti-CRTC3 polyclonal sheep IgG conjugated to Protein G Agarose resin. Following elution from the antibody-resin, total cell lysate (20 µg protein) and IP samples were resolved via SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were subjected to immunoblotting using the indicated antibodies.

Therefore, it was important to examine the efficacy of both HG-9-91-01 and MRT199665 in U2OS human osteosarcoma cells. However, it proved difficult to adequately detect CRTC3 phosphorylation directly in total cell lysates via SDS-PAGE and Western immunoblotting and as a result it was necessary to immunoprecipitate CRTC3 from total cell lysates prior to

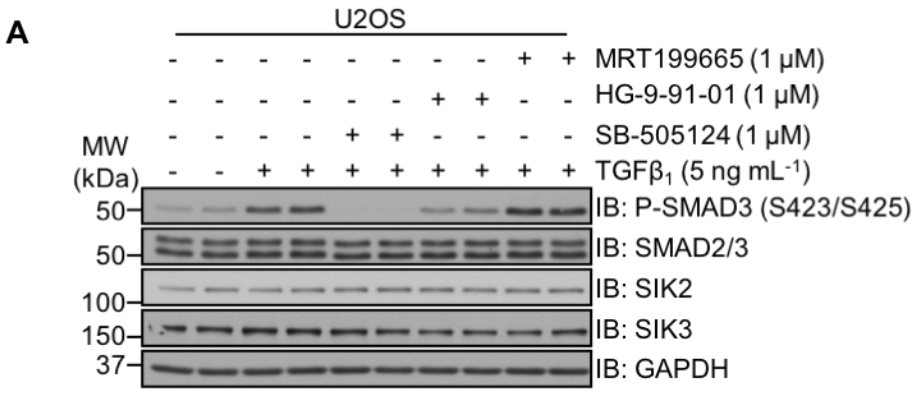
performing SDS-PAGE in order to do so. Incubation of wild type U2OS osteosarcoma cells with either HG-9-91-01 (0.5 μM) or MRT199665 (1 μM) resulted in a substantial reduction in phosphorylation of CRTC3 at serine 370 compared with DMSO control treated cells, indicating that both compounds were effectively inhibiting SIKs at the respective concentrations used (figure 3.2E).

3.2.4 Analysis of SIK inhibitors on receptor-mediated SMAD3 phosphorylation

The development of cell-permeable small-molecule inhibitors targeting protein kinases has helped to delineate the physiological substrates and hence the cellular functions of these enzymes in the study of cell signalling. However, the degree of target selectivity of an inhibitor represents one of the principal caveats of using small-molecule inhibitors as research tools. A number of studies have performed *in vitro* profiling of some commonly used inhibitors against panels of protein kinases in order to investigate the specificities of these compounds (Davies *et al.*, 2000; Bain *et al.*, 2003). This research revealed that certain compounds that were reported to inhibit particular protein kinases also inhibited multiple other kinases and, in some cases, more potently than their intended target. Thus, it is critically important to consider the specificities of small-molecule inhibitors to avoid drawing erroneous conclusions.

Therefore, it was important to test whether the inhibition of the TGFβ-induced luciferase reporter activity that was observed upon treatment with the inhibitors HG-9-91-01 and MRT199665 (figure 3.2D) was a result of the off-target inhibition of the TGFβ receptor kinases upstream of SMAD2/SMAD3 phosphorylation. Wild type U2OS osteosarcoma cells were incubated with SB-505124, HG-9-91-01, MRT199665 or DMSO and stimulated with recombinant human $TGF\beta_1$ (5 ng mL^{-1}) for 1 hour. As expected, incubation of cells with the TGFβ type I receptor inhibitor SB-505124 completely abolished the receptor-mediated phosphorylation of SMAD3. However, the HG-9-91-01 compound also resulted in partial attenuation of receptor-mediated SMAD3 phosphorylation, whereas cells treated with MRT199665 exhibited SMAD3 phosphorylation levels comparable to that observed in DMSO control treated cells (*figure 3.2-D*). This suggested that HG-9-91-01 could potentially inhibit either the TGFβ type I or type II receptor kinase and therefore the ability of HG-9-91-01 and

MRT199665 to inhibit the TGFβ type I receptor TGFβRI (ALK5) was subsequently tested *in vitro*. At the three inhibitor concentrations analysed (0.1, 1.0 and 10 μM), HG-9-91-01 potently inhibited the catalytic activity of TGFβRI (figure 3.2F). However, in contrast, MRT199665 had no inhibitory effect on TGFβRI activity (figure 3.2F). Collectively, these results indicate that the inhibition of TGFβ-induced luciferase reporter activity by HG-9-91-01 observed in the pharmacological screen and subsequent validation experiments occurred due, in part, to the off-target inhibition of the TGFβ type I receptor kinases. Interestingly, MRT199665 displayed no inhibition of the TGFβ type I receptor *in vitro* and did not affect receptor-mediated phosphorylation of SMAD3, however it consistently blocked TGFβ-induced luciferase transcriptional reporter activity. This resulted in the hypothesis that the inhibitory effect of MRT199665 on TGFβ-dependent gene transcription occurred downstream of the receptor-mediated phosphorylation and activation of SMAD2 and SMAD3. For these reasons, the HG-9-91-01 compound was not used in any subsequent experiments and the attention was focused upon validating the effect of MRT199665 on TGFβ signalling.



B

TGFβRI (ALK5)	Inhibitor conc. (μM)		
	0.1	**1.0**	**10.0**
SIK inhibitor	% Activity (± SD)	% Activity (± SD)	% Activity (± SD)
HG-9-91-01	4.8 ± 0.82	3.3 ± 0.13	3.0 ± 0.02
MRT199665	102.2 ± 4.57	94.3 ± 0.29	97.7 ± 1.22

Figure 3.2F. HG-9-91-01 inhibits the TGFβ receptor-mediated phosphorylation of SMAD3
(A) Wild-type U2OS osteosarcoma cells were incubated with SB-505124, HG-9-91-01 and MRT199665 at the concentrations indicated or equivalent volume of DMSO and stimulated

with recombinant human TGFβ_1 (5 ng mL^{-1}) for 1 hour prior to cell lysis. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. Immunoblots are representative of three independent experiments. **(B)** *In vitro* kinase assay analysis of recombinant constitutively active TGFβRI (ALK5) in the presence of either HG-9-91-01 or MRT199665 at three different concentrations (0.1, 1.0 and 10.0 µM). Results indicate mean percentage activity remaining and standard deviation (SD) resulting from three independent experiments. The *in vitro* kinase assay experiments were performed by the MRC PPU International Centre for Kinase Profiling at the University of Dundee (http://www.kinase-screen.mrc.ac.uk).

3.2.5 *In vitro* protein kinase analysis of SIK isoforms against SMAD2 and SMAD3

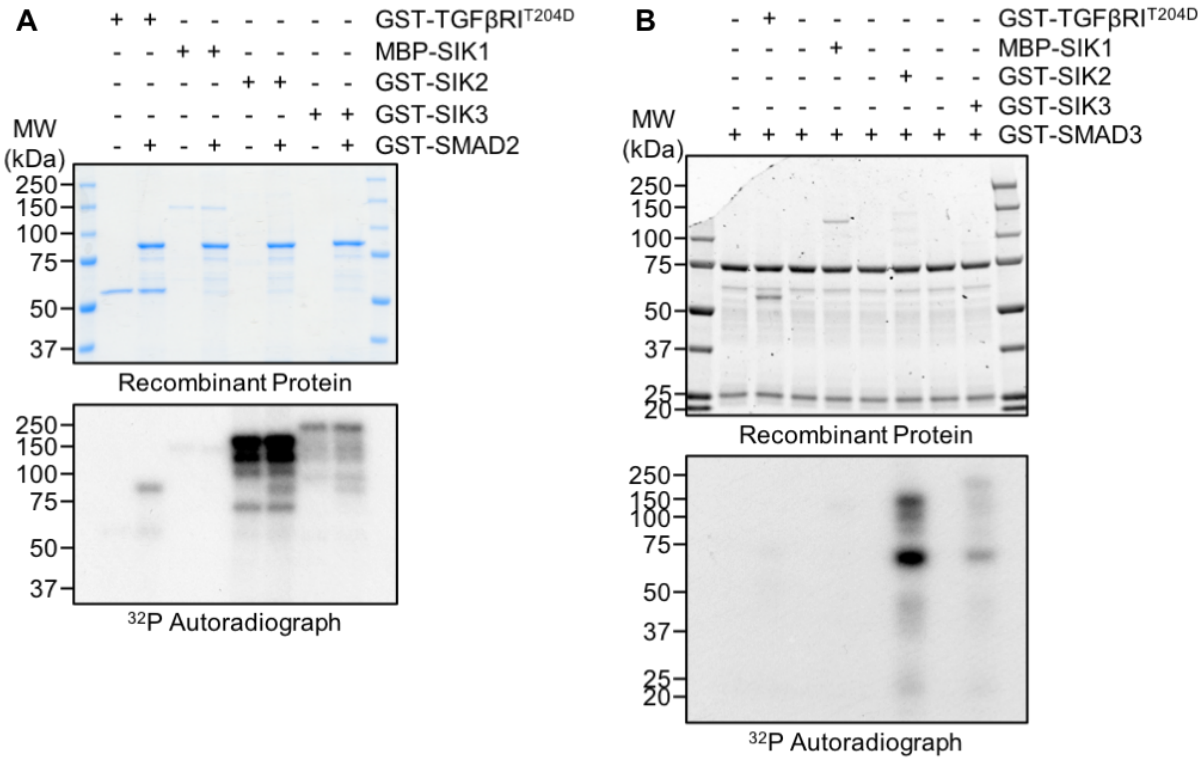
Following on from the identification of SIKs as potential regulators of TGFβ-induced luciferase reporter activity, we wanted to investigate whether SMAD2 or SMAD3 were potential substrates for any of the SIK isoforms. Therefore, we performed *in vitro* protein kinase assays using baculovirus-expressed recombinant MBP-SIK1 (2-783), GST-SIK2 (2-926) and GST-SIK3 (2-1369) protein kinases and bacterially expressed (*Escherichia coli*) GST-SMAD2 (1-467) or GST-SMAD3 (1-425). As a positive control for SMAD phosphorylation, the baculovirus-expressed recombinant cytoplasmic protein kinase domain (residues 200-501) of the type I TGFβ receptor (TGFβRI, alternatively known as ALK5) with a single point mutation (T204D) was included. The threonine (Thr, T) 204 residue is located at the carboxyl-terminal end of the GS domain adjacent to the canonical start of the kinase domain. Phosphorylation of multiple serine and threonine residues within the GS domain of TGFβRI by the type II receptor kinase (TGFβRII) is required for TGFβRI activation and subsequent downstream signalling. Therefore, substitution of Thr204 with an acidic aspartic acid residue (Asp, D) simulates negatively charged phosphate groups and results in a constitutively activated receptor. Consequently, TGFβRI^{T204D} displays enhanced kinase activity *in vitro* and signals independent of both TGFβ ligand and TGFβRII (Wieser, Wrana and Massagué, 1995).

Phosphorylation of SMAD2 was observed in the presence of TGFβRI^{T204D}, GST-SIK2 and to a lesser extent GST-SIK3. However, the level of SMAD2 phosphorylation was substantially less than the autophosphorylation observed for both GST-SIK2 and GST-SIK3. Unfortunately, the purified recombinant MBP-SIK1 protein displays negligible *in vitro* kinase activity, as observed by very weak autophosphorylation and also confirmed by further analysis conducted by the MRC PPU International Centre for Kinase Profiling at the University of

Dundee. Therefore, it proved challenging to ascertain whether the SIK1 isoform could phosphorylate either SMAD2 or SMAD3 *in vitro*.

Surprisingly, when *in vitro* kinase assays were performed using GST-SMAD3 as the substrate, SMAD3 phosphorylation was not observed in the presence of TGFβRI^{T204D} under the experimental conditions employed (figure 3.2G). Subsequent repeat experiments using different preparations of TGFβRI^{T204D} yielded the same observation. It is possible that the purified recombinant GST-SMAD3 does not fold correctly, or that the GST tag somehow interferes with the ability of the purified recombinant TGFβRI^{T204D} to phosphorylate the SMAD3 carboxy-terminal SSXS motif. Interestingly however, robust phosphorylation of GST-SMAD3 in the presence of GST-SIK2, and to a lesser extent in the presence of GST-SIK3 was observed (figure 3.2G-B). Furthermore, the level of SMAD3 phosphorylation was substantially higher than the level of autophosphorylation observed for both GST-SIK2 and GST-SIK3 (figure 3.2G-B). Additionally, the inclusion of the small-molecule SIK inhibitor HG-9-91-01 at 1 µM completely inhibited the ability of both GST-SIK2 and GST-SIK3 to phosphorylate SMAD3 (figure 3.2G-C). This raised the possibility that SMAD3 (and perhaps SMAD2, to a lesser degree) are substrates of SIKs and in particular the SIK2 isoform. However, these *in vitro* kinase assays were conducted using recombinant SMAD2 and SMAD3, both of which contained an amino-terminal GST-tag. Glutathione S-transferase (GST) is composed of 222 amino acid residues (with an approximate molecular weight of 26 kDa) and is thus considered to be a relatively large affinity tag. It is therefore conceivable that the phosphorylation that was observed in these assays was a result of phosphorylation of the GST tag and not the SMAD protein itself. Both the recombinant GST-SMAD2 and GST-SMAD3 contained a PreScission Protease cleavage sequence to enable the removal of the GST tag. The PreScission Protease specifically cleaves between the Gln and Gly residues of the amino acid recognition sequence Leu-Glu-Val-Leu-Phe-Gln/Gly-Pro (LEVLFQ/GP) (Cordingley *et al.*, 1990). When subsequent *in vitro* protein kinase assays were conducted using proteolytically cleaved full-length recombinant SMAD3 protein, SIK2 and SIK3 recombinant kinase were still able to robustly phosphorylate SMAD3, indicating that they were indeed phosphorylating SMAD3, and not the GST affinity tag, *in vitro*. Moreover, as observed in previous experiments, the inclusion of the potent SIK small-molecule inhibitor HG-9-91-01 completely abolished the ability of SIK2 and SIK3 to phosphorylate SMAD3, providing evidence that the SMAD3 phosphorylation is occurring due to SIKs and not a contaminating protein kinase. Identifying the residues of

SMAD3 that are subject to SIK-mediated phosphorylation and discerning the functional consequences is the principal focus of my current research.


A
GST-TGFβRI^T204D
MBP-SIK1
GST-SIK2
GST-SIK3
GST-SMAD2
MW (kDa)
250
150
100
75
50
37
Recombinant Protein
32P Autoradiograph
B
GST-TGFβRI^T204D
MBP-SIK1
GST-SIK2
GST-SIK3
GST-SMAD3
MW (kDa)
250
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Recombinant Protein
32P Autoradiograph

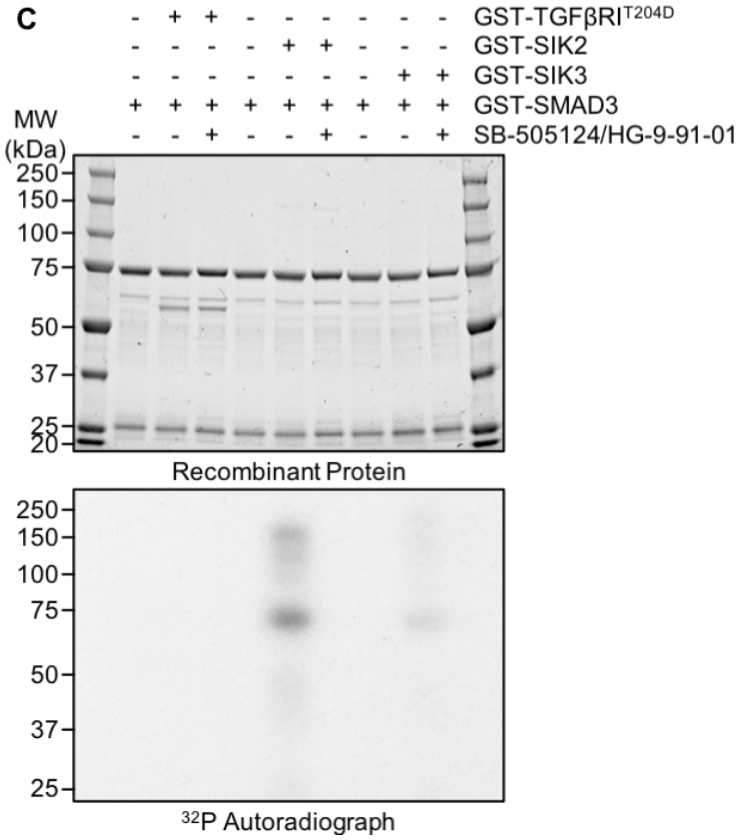

C
GST-TGFβRI^T204D
GST-SIK2
GST-SIK3
GST-SMAD3
SB-505124/HG-9-91-01
MW (kDa)
250
150
100
75
50
37
25
20
Recombinant Protein
32P Autoradiograph

Figure 3.2G. *In vitro* kinase assay analysis of SIK isoforms against GST-SMAD2 and GST-SMAD3

(A) *In vitro* kinase assay using recombinant human TGFβRI^{T204D} (residues 200-501), MBP-SIK1 (residues 2-783), GST-SIK2 (residues 2-926) and GST-SIK3 (residues 2-1369) protein kinases (200 ng kinase per reaction) and GST-SMAD2 (residues 1-467) (2 μg substrate per reaction). Kinase assay reactions were performed using 1x kinase assay buffer containing 0.1 mM [γ32P]-ATP (500 cpm pmol^{-1}) for 30 minutes at 30°C. Reactions were terminated via the addition of NuPAGE 4x LDS sample buffer containing 8% (v/v) 2-ME and incubation at 95°C for 5 minutes. Samples were resolved via SDS-PAGE and polyacrylamide gel subsequently stained with Coomassie protein stain before radioactivity was analysed by autoradiography. **(B)** *In vitro* kinase assay using recombinant human TGFβRI^{T204D} (residues 200-501), MBP-SIK1 (residues 2-783), GST-SIK2 (residues 2-926) and GST-SIK3 (residues 2-1369) protein kinases (200 ng kinase per reaction) and GST-SMAD3 (residues 1-425). Kinase reactions were conducted using the same experimental procedure as described above. **(C)** *In vitro* kinase assay using recombinant human TGFβRI^{T204D} (residues 200-501), GST-SIK2 (residues 2-926) and GST-SIK3 (residues 2-1369) protein kinases (200 ng kinase per reaction) and GST-SMAD3 (residues 1-425). In addition, either SB-505124 or HG-9-91-01 small-molecule kinase inhibitors (both at 1 μM concentration) were included as positive controls. Kinase reactions were conducted using the same experimental procedure as described previously.

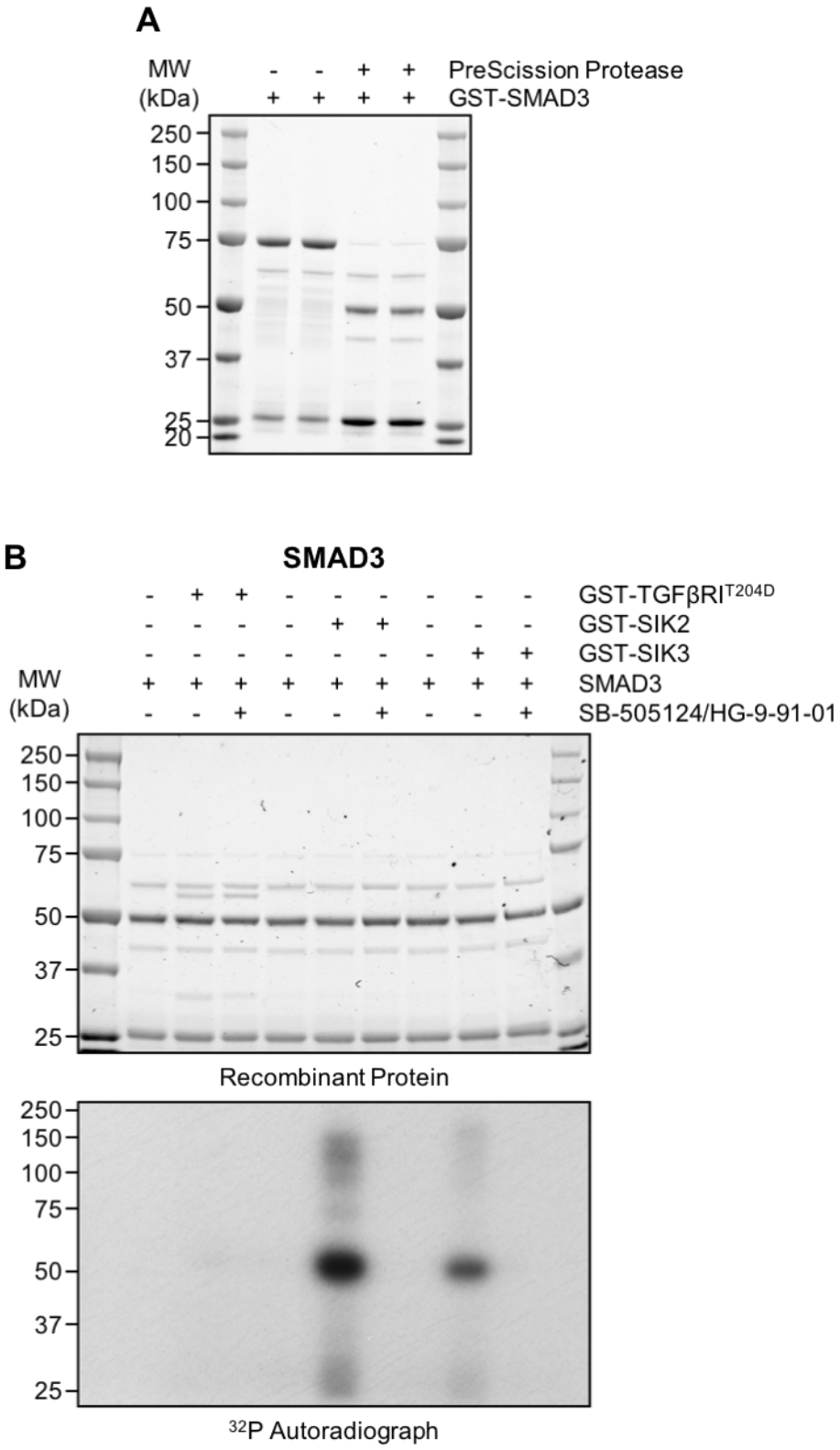


Figure 3.2H. *In vitro* kinase assay analysis of recombinant SIK2 and SIK3 against SMAD3
(A) The GST affinity tag was removed from recombinant GST-SMAD3 using PreScission Protease. Reaction was performed using 1:100 (weight) ratio of protease to substrate and incubated overnight at 4°C with continuous rotation. **(B)** *In vitro* kinase assay using recombinant human TGFβRI^{T204D} (residues 200-501), GST-SIK2 (residues 2-926) and GST-SIK3

(residues 2-1369) protein kinases (200 ng kinase per reaction) and cleaved recombinant SMAD3 (residues 1-425). In addition, either SB-505124 or HG-9-91-01 small-molecule kinase inhibitors (both at 1 μM concentration) were included as positive controls. Kinase reactions were conducted using the same experimental procedure as described previously.

3.2.6 MRT199665 does not affect the TGFβ-dependent nuclear accumulation of SMAD2 or SMAD3

The regulation of gene transcription mediated by TGFβ is critically dependent on the nuclear accumulation of activated SMAD transcriptional complexes. The steady-state nucleocytoplasmic distribution of R-SMADs was initially considered to be static, with R-SMADs residing exclusively in the cytoplasm in the absence of a signal and subsequently translocating into the nucleus upon detection of a TGFβ signal. In contrast to this, SMAD4 is distributed equally between the cytoplasm and nucleus, undergoing rapid and constant nucleocytoplasmic shuttling even in the absence of a TGFβ signal (Pierreux, Nicolás and Hill, 2000). However, upon further investigation, evidence also suggested that R-SMADs are undergoing continuous nucleocytoplasmic shuttling and require sustained receptor activation in order to maintain them in nucleus and facilitate TGFβ-dependent transcriptional regulation (Inman, Nicolás and Hill, 2002; Reguly and Wrana, 2003; Hill, 2009). As a result, the concentration of activated SMAD complexes present in the nucleus is dictated by the levels of activated receptor complexes in the cytoplasm (Inman, Nicolás and Hill, 2002).

Therefore, it was necessary to determine whether the MRT199665 compound was affecting SMAD nuclear translocation following TGFβ stimulation, as this may account for the inhibition of TGFβ-induced luciferase reporter activity observed in the previous experiments. Wild type U2OS osteosarcoma cells were incubated with either the TGFβ type I receptor inhibitor SB-505124, the SIK inhibitor MRT199665 or an equivalent volume of DMSO and stimulated with recombinant human $TGF\beta_1$ for 1 hour and subsequently fractionated into cytoplasmic and nuclear fractions. In DMSO control cells, phosphorylated SMAD2 and SMAD3 were exclusively present in the nuclear fractions, with enhanced levels observed following TGFβ stimulation (figure 3.2I). As expected, treatment of cells with SB-505124 dramatically reduced the levels of phosphorylated SMAD2 and SMAD3 in the nucleus following TGFβ stimulation (figure 3.2I). Consistent with previous experiments, MRT199665 did not affect the level of receptor-mediated SMAD2 or SMAD3 phosphorylation. Furthermore, the nuclear

translocation of SMAD2 and SMAD3 in response to TGFβ signals remained unaffected by treatment with MRT199665 (figure 3.2I). This data implied that the inhibitory effect of MRT199665 on TGFβ-induced luciferase reporter activity occurred following nuclear accumulation, at the level of gene transcription.

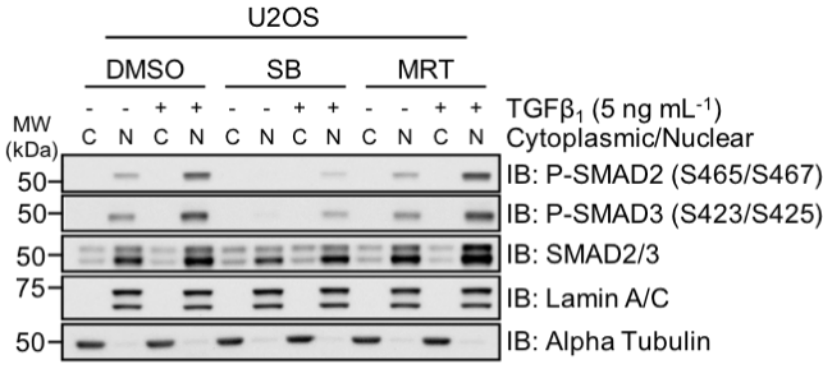


Figure 3.2I. MRT199665 does not affect the TGFβ-induced nuclear translocation of SMAD2 or SMAD3
Wild type U2OS osteosarcoma cells were incubated with SB-505124 (1 μM), MRT199665 (1 μM) or an equivalent volume of DMSO in the presence or absence of stimulation with recombinant human TGFβ$_1$ (5 ng mL^{-1}) for 1 hour prior to cell lysis. Cell lysates were separated into cytoplasmic and nuclear fractions, resolved via SDS-PAGE and transferred to nitrocellulose membranes. Membranes were subsequently subjected to immunoblotting with the indicated antibodies. Alpha tubulin and lamin A/C were used as markers for cytoplasmic and nuclear fractions respectively. Immunoblots are representative of two independent experiments.

3.2.7 Discussion

In the previous section 3.1, I reported that the small-molecule inhibitor screen performed in the endogenous TGFβ-dependent transcriptional reporter cell line resulted in the identification of HG-9-91-01, an ATP-competitive inhibitor of SIK isoforms, as a potential regulator of TGFβ signalling. Therefore, in this section, the further characterisation of SIK inhibition in the context of TGFβ signalling has been presented.

In addition to HG-9-91-01, MRT199665, another ATP-competitive small-molecule inhibitor that has been used to inhibit the catalytic activity of SIK isoforms was also utilised. Although HG-9-91-01 and MRT199665 are structurally unrelated inhibitors, they are capable of potently inhibiting SIKs *in vitro* (<100 nM IC$_{50}$ values). Therefore, the effect of both of these

inhibitors on the endogenous TGFβ transcriptional reporter cell line was subsequently analysed. Treatment of U2OS 2G transcriptional reporter cells with either HG-9-91-01 or MRT199665 resulted in the statistically significant attenuation of TGFβ-dependent luciferase reporter activity. Subsequently, it was important to demonstrate that both HG-9-91-01 and MRT199665 were effectively inhibiting SIK isoforms in U2OS osteosarcoma cells at the concentrations that were employed. In order to do this, the phosphorylation status of the validated physiological SIK substrate CRTC3, a transcriptional co-activator involved in CREB-dependent gene transcription, was therefore analysed. The incubation of U2OS cells with either HG-9-91-01 or MRT199665 promoted the dephosphorylation of CRTC3 at the SIK-dependent phosphorylation site serine 370. Collectively, this data indicated that two structurally unrelated kinase inhibitors of SIKs were both capable of mediating the attenuation of the endogenous TGFβ-dependent transcriptional reporter at concentrations that were effective at inhibiting SIK isoforms in cultured cells. However, one of the principal caveats of using small-molecule inhibitors in experimental research is that they are associated with the 'off-target' inhibition of protein kinases other than those for which they are intended to inhibit. As a consequence, it is vital to conduct experiments with the appropriate controls in order to avoid the erroneous interpretation of experimental data. Although in this section it has been demonstrated that two structurally unrelated kinase inhibitors exert the same effect on TGFβ transcriptional reporter, the data is not sufficient to ascertain whether this is through inhibition of SIK isoforms. For example, although HG-9-91-01 is selective for SIK isoforms amongst the AMPK-related family of kinases, it is also capable of mediating the inhibition of other protein kinase such as discoidin domain-containing receptor 2 (DDR2), ephrin type A and type B receptors, fibroblast growth factor receptor 1 (FGFR1), receptor interacting serine/threonine-protein kinase 2 (RIPK2), protein-tyrosine kinase 6 (PTK6, otherwise referred to as breast tumour kinase; BRK) and tyrosine-protein kinase Yes (YES1). Moreover, in addition to mediating the inhibition of SIK isoforms, MRT199665 is also capable of potently inhibiting the AMPK catalytic subunits, the four MARK isoforms and NUAK1 *in vitro*. Consequently, the observation that HG-9-91-01 and MRT199665 can attenuate the TGFβ-dependent induction of luciferase activity in the endogenous transcriptional reporter cell line may be a consequence of the 'off-target' inhibition of either the TGFβ type I or type II serine-threonine receptor kinases. The data obtained from subsequent experiments

demonstrated that HG-9-91-01 is capable of completely abrogating the receptor-mediated phosphorylation of SMAD3 and is a potent inhibitor of the TGFβ type I receptor ALK5 *in vitro*. Therefore, unfortunately, it was not possible to deploy HG-9-91-01 in any subsequent experiments involving TGFβ signalling performed as part of this doctoral project.

Although MRT199665 did not inhibit the TGFβ type I receptor nor affect SMAD2 and SMAD3 phosphorylation, another possible explanation for the consistent observation that MRT199665 attenuates TGFβ-dependent luciferase reporter activity is that it may prevent the nuclear translocation of the TGFβ-activated SMAD transcription factors SMAD2 and SMAD3. However, from the experiments presented in this section, it has been demonstrated that MRT199665 does not impede the ability of either SMAD2 or SMAD3 to translocate into the nucleus in response to TGFβ stimulation. Therefore, the effect of MRT199665 appears to occur further downstream the signalling pathway, potentially at the level of transcriptional regulation. This may occur by either preventing the association of activated SMAD transcriptional complexes with regulatory DNA sequences or by interfering with the interaction of SMAD complexes with obligate transcriptional co-regulators. In future research, I aim to investigate whether incubation of cells with MRT199665 influences the ability of activated SMAD transcriptional complexes to associate with regulatory DNA sequences by conducting chromatin immunoprecipitation (ChIP) experiments. The ChIP assay would involve the immunoprecipitation of SMAD2 and SMAD3 from cell lysates following stimulation with recombinant TGFβ and determining their ability to bind to regulatory sequences within the *PAI-1* promoter region.

From the experiments presented thus far, it is important to state that the effect of MRT199665 on TGFβ-induced transcriptional responses may well be the result of inhibition of protein kinases other than SIKs. The further validation of SIKs in the modulation of TGFβ-mediated transcriptional induction will be addressed in subsequent sections.

3.3 VALIDATION OF SIK INHIBITION AS A REGULATOR OF TGFβ-INDUCED GENE TRANSCRIPTION

3.3.1 Introduction

The data presented in the preceding section demonstrated that the small-molecule kinase inhibitor MRT199665 is capable of attenuating the induction of the endogenous TGFβ-dependent luciferase reporter activity and moreover, this attenuation occurred without affecting receptor-mediated R-SMAD phosphorylation or the nuclear translocation of activated SMAD complexes. This therefore results in the hypothesis that the effect of the MRT199665 kinase inhibitor is transpiring further downstream the pathway at the level of transcriptional regulation, however as mentioned in the previous discussion section, future experiments are aimed to test this hypothesis. In this section, evidence is presented that demonstrates that the effect of MRT199665 is not restricted to the endogenous TGFβ-dependent transcriptional reporter cell line and that the inhibitor mediates the attenuation of TGFβ-induced transcriptional upregulation of the physiological target gene *PAI-1* in two different human cancer cell lines. Furthermore, the following data also indicates that MRT199665 attenuates the transcriptional induction of additional TGFβ target genes, indicating that it is not a gene-dependent observation and may be prevalent in other TGFβ-mediated transcriptional responses.

3.3.2 The small-molecule inhibitor MRT199665 suppresses TGFβ-induced target gene expression

The small-molecule inhibitor screen and subsequent validation experiments that were performed demonstrated that MRT199665 could efficiently attenuate the activity of an endogenous TGFβ-dependent transcriptional reporter. Furthermore, this attenuation occurred in the absence of receptor kinase inhibition, diminished receptor-mediated phosphorylation of the intracellular mediators SMAD2 and SMAD3 or perturbation of SMAD nuclear translocation. The small-molecule inhibitor screen and initial validation experiments conducted employed an endpoint luciferase assay as the detection method. Bioluminescence is a frequently used detection technique in high-throughput screening (HTS) assays for

chemical biology and drug discovery applications (Thorne, Inglese and Auld, 2010). One of the principal advantages for the utilisation of luminescence-based assays is that, unlike fluorescence-based detection systems, they do not require excitation light energy. Therefore, they provide a sensitive assay detection technique with a high signal-to-background ratio. However, the main limitation of luminescence-based reporter assays in HTS is the potential for compounds to modulate the enzymatic activity of luciferase, resulting in experimental artefacts that can interfere with the interpretation of assay results (Thorne, Inglese and Auld, 2010). In cell-based assays, the enzymatic activity of luciferase can be confounded by compounds that inhibit the luciferase enzyme, although the mode of action that results in enzyme inhibition can vary. For example, the compound resveratrol has been investigated for its apparent therapeutic potential as a cardioprotective, neuroprotective and chemopreventive agent, in addition to the observation that it can extend the lifespan of a number of model organisms (Baur and Sinclair, 2006). Despite this, the molecular mechanism and signalling networks by which resveratrol may mediate these potential therapeutic effects remain uncertain. Research employing luciferase-based reporter gene assays to identify molecular pathways regulated by resveratrol may be undermined by the reported observation that resveratrol can potently inhibit the enzymatic activity of firefly luciferase (Bakhtiarova *et al.*, 2006). Furthermore, research has also demonstrated that firefly luciferase is susceptible to the phenomenon of ligand-induced protein stabilisation. Compounds that function as effective competitive inhibitors of firefly luciferase can reduce the rate of protein degradation *in vivo*, thereby resulting in an accumulation of the reporter enzyme and increased luminescence signal upon detection (Thompson, Hayes and Lloyd, 1991). Therefore, considering the potential for compounds to modulate the enzymatic activity of luciferase, one possible explanation for the attenuation of luciferase signal observed following treatment with MRT199665, is that the compound may be inhibiting the luciferase enzyme. As previously discussed, the endogenous TGFβ-dependent transcriptional reporter cell line developed in the lab contained a dual-reporter cassette containing both firefly luciferase enzyme and GFP. Therefore, using this cell line, we tested whether the MRT199665 compound also attenuated the protein expression of GFP following TGFβ stimulation. U2OS 2G transcriptional reporter cells were incubated with either DMSO, SB-505124 or MRT199665 and stimulated with recombinant human $TGF\beta_1$ (5 ng mL^{-1}) for 6 hours. In DMSO control

treated cells, TGFβ induced receptor-mediated phosphorylation of both SMAD2 and SMAD3, resulting in a concomitant induction of GFP protein expression (figure 3.3A-A). This induction was dependent on TGFβ, as treatment with the TGFβ type I receptor kinase inhibitor SB-505124 abrogated both SMAD phosphorylation and GFP expression (figure 3.3A-A). Consistent with previous experiments, treatment of cells with MRT199665 did not affect the level of receptor-mediated SMAD2/SMAD3 phosphorylation in response to TGFβ, however in these cells, the TGFβ-mediated induction of GFP expression was completely prevented (figure 3.3A-A). In addition, we investigated the effect of MRT199665 on the TGFβ-induced expression of endogenous PAI-1 in wild type U2OS osteosarcoma cells. TGFβ induces a 2-fold induction in the relative expression of PAI-1 mRNA and this induction is prevented when cells are treated with SB-505124 (figure 3.3A-B). Treatment of U2OS cells with MRT199665 significantly attenuated both PAI-1 mRNA and protein expression in response to TGFβ (figure 3.3A-B). Importantly, this datum indicates that the effect of the MRT199665 small-molecule inhibitor on the endogenous TGFβ-responsive luciferase reporter is not a consequence of the compound modulating the activity of the luciferase enzyme. Furthermore, it also provides further evidence to suggest that MRT199665 exerts it effect at the level of target gene transcription.

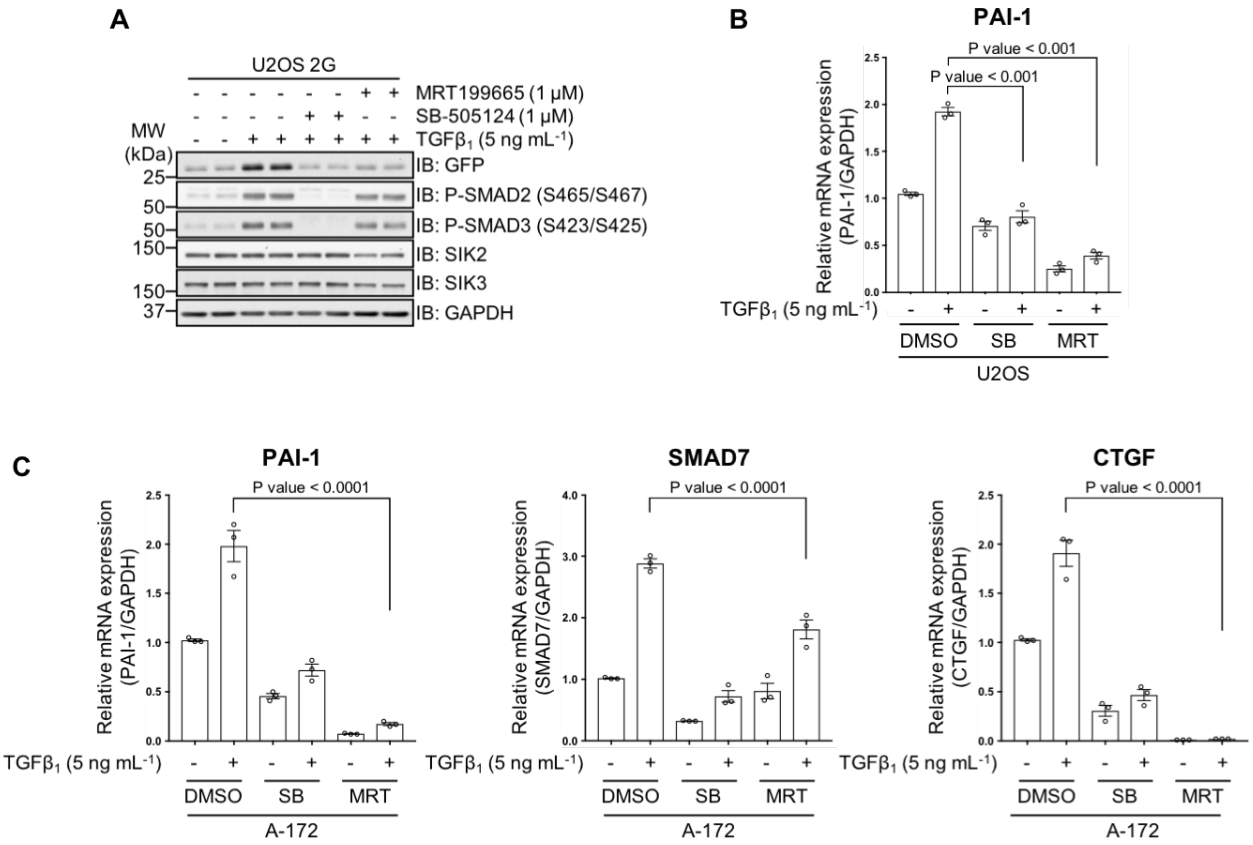


Figure 3.3A. MRT199665 attenuates the expression of TGFβ-dependent target genes in multiple human cancer cell lines
(A) U2OS 2G transcriptional reporter cells were incubated with SB-505124 or MRT199665 at the concentrations indicated, or an equivalent volume of DMSO and stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 6 hours prior to cell lysis. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. Immunoblot is representative of two independent experiments. **(B)** Wild-type U2OS human osteosarcoma cells were incubated with SB-505124 or MRT199665 (both inhibitors used at 1 µM concentration), or an equivalent volume of DMSO and stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 6 hours prior to lysis and RNA isolation. Complementary DNA (cDNA) was synthesised from the isolated RNA and RT-qPCR analysis performed for the indicated TGFβ target gene. The statistical analysis was performed on data from three independent experiments. **(C)** Wild-type A-172 human glioblastoma cells were incubated with SB-505124 or MRT199665 (both inhibitors used at 1 µM concentration), or an equivalent volume of DMSO and stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 6 hours prior to lysis and RNA isolation. Complementary DNA (cDNA) was synthesised from the isolated RNA and RT-qPCR analysis performed for the indicated TGFβ target genes. Statistical analysis was performed on data obtained from three biological replicates. The experiment was conducted by Dr. Polyxeni Bozatzi.

Subsequently, experiments were performed to investigate whether MRT199665 could abrogate TGFβ-induced expression of PAI-1 within a different cellular context. Therefore, the A-172 human glioblastoma cell line, which have previously been employed by other research groups to interrogate TGFβ signalling (Seystahl *et al.*, 2015; Jun *et al.*, 2017; Kit Leng Lui *et al.*, 2017) was utilised. The upregulation of PAI-1 expression was robustly upregulated in response to TGFβ stimulation. Similar to U2OS osteosarcoma cells, the mRNA expression of PAI-1 increases 2-fold following 6 hours of incubation with recombinant human TGFβ_1 (figure 3.3A-C). Consistent with the effect observed on TGFβ-induced PAI-1 expression in U2OS cells, co-treatment of A-172 cells with 1 µM MRT199665 and TGFβ_1 for 6 hours also robustly attenuated PAI-1 mRNA expression induced by TGFβ stimulation (figure 3.3A-C).

Furthermore, it was also important to ascertain whether or not the effect of MRT199665 on TGFβ-dependent gene transcription was restricted to PAI-1 or could MRT199665 treatment also modulate the expression of other known TGFβ target genes. Therefore, using the TGFβ-responsive A-172 human glioblastoma cell line, the effect of

MRT199665 on the transcription of two other well-characterised TGFβ target genes, SMAD7 and connective tissue growth factor (CTGF), was investigated. As discussed in section 1.2.3, SMAD7 functions as an inhibitory SMAD protein to antagonise TGFβ signalling via numerous different mechanisms including competing with R-SMADs for binding to the activated TGFβ type I receptor, recruiting E3 ubiquitin protein ligases to the activated receptor complex to mediate its proteasomal degradation and also facilitating the dephosphorylation of the activated TGFβ type I receptor through recruitment of protein phosphatase 1 (PP1) (Kavsak *et al.*, 2000; Shi *et al.*, 2004; Valdimarsdottir *et al.*, 2006). SMAD7 is a direct transcriptional target of SMAD-dependent TGFβ signalling (Nakao *et al.*, 1997; Afrakhte *et al.*, 1998; Nagarajan *et al.*, 1999; Denissova *et al.*, 2000) and thus functions in a negative feedback mechanism. Stimulation of A-172 glioblastoma cells with TGFβ_1 for 6 hours induced an approximately 3-fold induction in the mRNA expression of SMAD7 that was efficiently attenuated when cells were co-incubated with SB-505124 (figure 3.3A-C). As observed with TGFβ-mediated induction of PAI-1 mRNA expression, co-incubation with MRT199665 attenuated TGFβ-induced transcriptional upregulation of SMAD7 (figure 3.3A-C). However, the attenuation of SMAD7 mRNA expression by MRT199665 only occurs partially in this cell line, in contrast to the near-complete attenuation observed for PAI-1 mRNA expression.

3.3.3 Discussion

The data presented in this section has demonstrated that the small-molecule kinase inhibitor MRT199665 is able to attenuate TGFβ-dependent transcription of multiple different target genes in two different human cancer cell lines. Although the observed transcriptional attenuation mediated by MRT199665 is experimentally robust, experiments involving the expression of inhibitor-resistant mutant SIK isoforms are required in order to ascertain that the observed effects are occurring through inhibition of the SIKs and not through the inhibition of other protein kinases. Experiments analysing the effect of other structurally unrelated small-molecule inhibitors of SIK isoforms are presented in the subsequent section 3.5.

The observation that MRT199665 attenuates the transcription of three different TGFβ target genes (*PAI-1*, *SMAD7* and *CTGF*) indicates the mechanism by which the MRT199665

compound exerts its suppressive effect may be similar across numerous TGFβ-mediated transcriptional responses. One of the principal means by which to test this hypothesis would be to conduct RNA sequencing (RNA-Seq) in order to analyse the entire transcriptome, and specifically the TGFβ-dependent transcriptome, in a particular cellular context. This would hopefully provide an insight into whether the effects of MRT199665 treatment are specific to a subset of TGFβ target genes or whether it is universal to TGFβ-dependent gene transcription.

3.4 GENETIC EVIDENCE FOR THE INVOLVEMENT OF SIK ISOFORMS IN THE MODULATION OF TGFβ SIGNALLING

3.4.1 Introduction

The data presented thus far in this thesis project has demonstrated that the small-molecule kinase inhibitor MRT199665 is capable of modulating the TGFβ-mediated transcriptional regulation of specific target genes in multiple human cancer cell lines. Although small-molecule kinase inhibitors are invaluable experimental tools which can aid in deciphering the cellular functions of protein kinases, their use is accompanied with substantial limitations (discussed in further detail in the relevant sections), the principal of which is the 'off-target' inhibition of kinases other than those for which they are intended to inhibit. Consequently, in order to obtain robust data, it is imperative to demonstrate that any observed effects can be corroborated with alternative experimental means of inactivating the protein kinases of interest. Therefore, in the following section, evidence will be presented demonstrating the effect of modulating the upstream activating kinase of SIKs, RNAi-mediated depletion of SIK protein levels and the genetic inactivation of SIK isoforms on specific TGFβ-dependent transcriptional responses.

3.4.2 Overexpression of catalytically active LKB1 potentiates the TGFβ-mediated induction of PAI-1 expression

In order to provide genetic evidence for the function of SIK isoforms in the TGFβ pathway regulation, I utilised the HeLa human cervical adenocarcinoma cell line which do not express endogenous LKB1. As previously discussed, the serine-threonine protein kinase LKB1, in complex with its regulatory subunits STRADα/β and MO25α/β, functions as a master upstream regulatory kinase by phosphorylating and activating AMPK along with 12 members of the AMPK-related family including all three SIK isoforms (Hawley *et al.*, 2003; Hong *et al.*, 2003; Woods *et al.*, 2003; Lizcano *et al.*, 2004; Shaw *et al.*, 2004; Shackelford and Shaw, 2009). Therefore, normal HeLa cells which lack endogenous LKB1 expression have been extensively used in order to investigate the cellular functions of LKB1 and the downstream protein kinases that LKB1 activates. It has been proposed that the undetectable levels of LKB1 mRNA

observed in HeLa cells is a result of epigenetic inactivation caused by hypermethylation of the *LKB1* promoter region (Tiainen, Ylikorkala and Mäkelä, 1999). Subsequent research also reported the occurrence of large homozygous deletions within the *LKB1* gene locus of HeLa cells, resulting in the complete loss of full-length LKB1 mRNA transcript and protein expression (Wingo *et al.*, 2009; McCabe *et al.*, 2010). The loss of LKB1 expression in HeLa cells may contribute to the highly proliferative phenotype of these cells as expression of exogenous LKB1 exerted a growth suppressive effect (Tiainen, Ylikorkala and Mäkelä, 1999).

Importantly for the purposes of this thesis project, in normal LKB1-deficient HeLa cells or in HeLa cells expressing a catalytically inactive form of LKB1, the kinase activities of SIK1, SIK2 (alternatively referred to as QIK) and SIK3 (alternatively referred to as QSK) were found to be significantly reduced (20- to 40-fold) compared with HeLa cells expressing wild-type LKB1 (Lizcano *et al.*, 2004). In order to generate a catalytically inactive form of LKB1, the aspartic acid residue of the DFG motif was mutated to an alanine residue (D194A). The DFG motif is situated within the protein kinase domain and is involved in the coordination of an Mg^{2+} ion required for catalytic activity. Thus, mutation of the invariant aspartic acid to alanine disrupts this function and consequently renders LKB1 inactive (Guldberg *et al.*, 1999; Sapkota *et al.*, 2001; Boudeau *et al.*, 2003, 2004; Woods *et al.*, 2003; Lizcano *et al.*, 2004; Shaw *et al.*, 2004; Scott *et al.*, 2007; Fogarty and Hardie, 2009).

When normal LKB1-deficient HeLa cells were stimulated with recombinant human $TGF\beta_1$ for 6 hours, the mRNA expression of the target gene *PAI-1* increased approximately 1.5-fold compared with unstimulated control cells. However, the stable overexpression of wild-type LKB1 ($LKB1^{WT}$) significantly enhanced the TGFβ-induced transcriptional upregulation of PAI-1 more than 7-fold compared with normal control HeLa cells (figure 3.4A-A). Moreover, even when comparing unstimulated normal LKB1-deficient and $LKB1^{WT}$ HeLa cells, PAI-1 mRNA expression was substantially elevated by the restoration of LKB1 protein expression (figure 3.4A-A). Crucially, the exogenous expression of a catalytically inactive LKB1 mutant ($LKB1^{D194A}$) failed to enhance either basal or TGFβ-induced PAI-1 transcript levels (figure 3.4A-A). Indeed, PAI-1 mRNA expression levels were virtually identical between normal LKB1-deficient HeLa cells and those expressing the kinase inactive $LKB1^{D194A}$ form (figure 3.4A-A). Furthermore, the observations at the transcript level were reiterated at the level of protein expression. PAI-1 protein expression was undetectable by immunoblotting in normal LKB1-deficient cells, even in the presence of TGFβ stimulation (figure 3.4A-B). By

contrast, basal levels of PAI-1 protein were weakly detected in LKB1^{WT} HeLa cells and stimulation of these cells with TGFβ substantially enhanced PAI-1 protein expression. As observed with mRNA expression, the kinase inactive LKB1^{D194A} mutant was incapable of enhancing either basal or TGFβ-induced PAI-1 expression (figure 3.4A-B).

Thus, this data provides initial genetic evidence that the protein kinase activity of SIK isoforms are involved in the modulation of TGFβ-dependent transcriptional regulation of the target gene PAI-1. However, there remains a number of additional crucial experiments to be conducted using these cell lines. Firstly, it would be important to demonstrate that the enhanced mRNA and protein expression of PAI-1 observed in the LKB1^{WT} HeLa cells could be attenuated by the incubation of these cells with the small-molecule kinase inhibitor of SIK isoforms MRT199665. Additionally, it would also be imperative to demonstrate that SIK catalytic activity is abrogated in the normal LKB1-deficient HeLa cells and the HeLa cells expressing the LKB1^{D194A} kinase inactive mutant. One would predict that the phosphorylation of the physiological SIK substrate CRTC3 at serine 370 is substantially reduced, and that this phosphorylation is enhanced upon the stable overexpression of LKB1^{WT}. Moreover, it would also be interesting to analyse other TGFβ target genes in this context and to investigate TGFβ-dependent gene transcription in the context of LKB1-deficient MEF cell lines, which have previously been generated (Clark *et al.*, 2012).

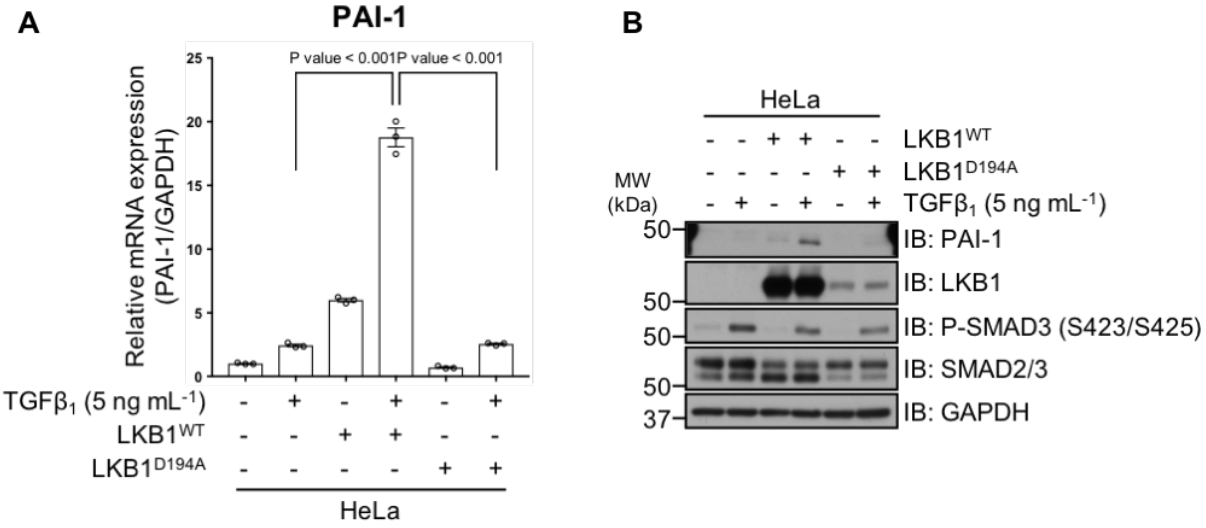


Figure 3.4A. The overexpression of wild type LKB1 potentiates the TGFβ-dependent transcriptional induction of PAI-1
(A) Wild type HeLa human cervical adenocarcinoma cells and HeLa cells stably overexpressing either $LKB1^{WT}$ or catalytically inactive $LKB1^{D194A}$ were stimulated with recombinant human $TGF\beta_1$ (5 ng mL^{-1}) for 6 hours prior to lysis and RNA isolation. Complementary DNA (cDNA) was synthesised from the isolated RNA and RT-qPCR analysis performed for the indicated TGFβ target gene (*PAI-1*). **(B)** Wild type HeLa human cervical adenocarcinoma cells and HeLa cells stably overexpressing either $LKB1^{WT}$ or catalytically inactive $LKB1^{D194A}$ were stimulated with recombinant human $TGF\beta_1$ (5 ng mL^{-1}) for 8 hours prior to cell lysis. Cell lysates (10 μg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. Immunoblot is representative of two independent experiments.

3.4.3 The RNAi-mediated depletion of SIK isoforms attenuates TGFβ-mediated upregulation of PAI-1 expression

To determine whether the modulation of TGFβ-induced PAI-1 expression was occurring through the SIKs, the protein expression of SIK isoforms was suppressed both individually and in combination using short interfering RNA (siRNA)-mediated gene silencing technology (Meister and Tuschl, 2004; Carthew and Sontheimer, 2009). The siRNA-mediated depletion of either SIK1 or SIK2 protein expression resulted in a partial reduction of TGFβ-induced PAI-1 protein expression compared to the expression levels observed in untransfected, transfection reagent control (RM; RNAiMAX) or non-targeting (NT) siRNA control cells (figure 3.4B-A). It is important to note that although the efficacy of SIK2 and SIK3 depletion could be analysed via immunodetection, due to the unavailability of an antibody that reliably detects endogenous human or murine SIK1, it was not possible to demonstrate the efficacy of SIK1 knockdown by immunoblotting. The depletion of the SIK3 isoform, either individually or in combination with SIK1 and SIK2, resulted in an even more pronounced suppression of TGFβ-mediated transcriptional upregulation of PAI-1 expression compared to control cell expression levels (figure 3.4B-A). This suggests that all three isoforms of SIKs may contribute to the transcriptional induction of PAI-1 expression in response to TGFβ stimulation.

Upon further investigation of SIK3 protein depletion, we observed that an almost complete silencing of SIK3 is required in order to observe the attenuation of TGFβ-upregulated PAI-1 expression (figure 3.4B-B). In cells in which the siRNA-mediated depletion

was only partial, the residual amount of SIK3 protein still present appeared to be sufficient to enable the equivalent PAI-1 upregulation as observed in control cells. Due to the inconsistencies in the efficacy of siRNA-mediated gene silencing of SIK isoforms, it was decided to focus upon alternative and more robust experimental methods of disrupting SIK function via genetic inactivation of kinase activity discussed in the subsequent section 3.4.4.

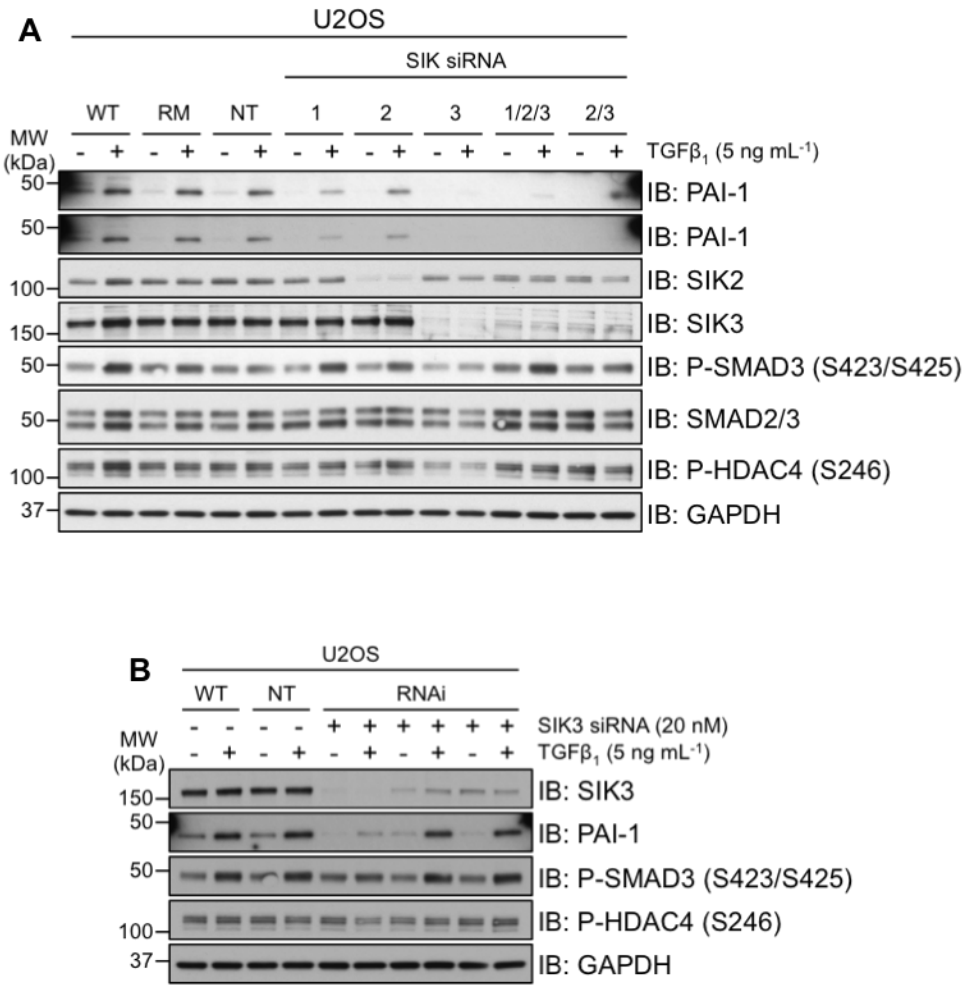


Figure 3.4B. The effect of RNAi-mediated silencing of SIK isoforms on TGFβ-dependent induction of PAI-1 expression

(A) U2OS human osteosarcoma cells were transiently transfected with either non-targeting (NT) siRNA oligonucleotide or siRNA oligonucleotide pools targeting the three SIK isoforms. Untransfected cells or cells transfection with only the lipid transfection reagent were included as experimental controls. Cells were incubated in the absence or presence of recombinant human TGFβ$_1$ (5 ng mL^{-1}) for 8 hours prior to cell lysis. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes

were subsequently subjected to immunoblotting with the indicated antibodies. Immunoblot is representative of three independent experiments. **(B)** U2OS osteosarcoma cells were transiently transfected with either non-targeting (NT) siRNA oligonucleotide or siRNA oligonucleotide pools targeting the SIK3 isoform. Cells were subsequently incubated in the absence or presence of recombinant human TGFβ_1 (5 ng mL^{-1}) for 8 hours prior to cell lysis. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. Immunoblot is representative of two independent experiments.

3.4.4 The genetic inactivation of SIK isoforms attenuates TGFβ-mediated transcriptional induction of PAI-1

In order to further establish the potential role of SIK isoforms in TGFβ signal transduction, the TGFβ-mediated transcriptional induction of the target gene *PAI-1* was analysed in mouse embryonic fibroblasts (MEFs) in which the wild type SIK2 and SIK3 proteins were replaced with catalytically inactive knock-in (KI) mutant forms. The development of the kinase inactive SIK KI mice was conducted by the research groups of Prof. Philip Cohen and Prof. Simon Arthur (respectively located in the MRC Protein Phosphorylation and Ubiquitylation Unit, and the Division of Cell Signalling and Immunology, both within the School of Life Sciences at the University of Dundee). The generation of primary MEF cell lines from the murine embryos was performed by Dr. Nicola J. Darling (P. Cohen research group). The constitutive kinase inactive SIK KI mice were created by conventional gene targeting technologies in the C57BL/6NTac genetic background by Taconic Biosciences. Targeting vectors were designed to introduce the required point mutation within the genomic locus of the relevant SIK isoform via homologous recombination (HR) in murine embryonic stem (ES) cells. Further information regarding the targeting strategy, design of targeting vectors and validation is available in the following publication (Darling *et al.*, 2017).

Firstly, it was important to determine that the TGFβ-mediated transcriptional upregulation of PAI-1 occurred in MEF cells. Therefore, wild-type MEFs were stimulated with recombinant human TGFβ_1 for different durations over a period of 24 hours and PAI-1 protein expression analysed via immunoblotting. As demonstrated in figure 3.4C-A, basal levels of PAI-1 protein is virtually undetectable via immunoblotting, however upon TGFβ stimulation, the expression of PAI-1 is robustly upregulated, with the highest protein expression detection after 12 hours of stimulation. Thus, MEF cells are amenable to TGFβ-mediated transcriptional

induction of the target gene *PAI-1* and therefore the impact of genetic inactivation of SIK isoforms on this transcriptional response was subsequently investigated.

As described in the preceding section, the SIKs, along with AMPK and other members of the AMPK-related family of kinases, are activated by LKB1-mediated phosphorylation of the conserved threonine residue within the activation loop (Lizcano *et al.*, 2004; Jaleel *et al.*, 2005) (figure 3.4C-B). Therefore, this residue was selected for mutation to an alanine residue as it is essential for the catalytic activity of SIKs (Lizcano *et al.*, 2004). The mice harbouring homozygous SIK1^{T182A} and SIK2^{T175A} genotypes were viable and fertile, and phenotypically indistinguishable from wild type mice whereas homozygous SIK3^{T163A} mice presented at sub-Mendelian ratios and were on average smaller compared with wild type or heterozygous counterparts. In addition to the generation of individual SIK KI mice, SIK1/SIK2 and SIK2/SIK3 double KI mice were also generated. As observed with SIK1 KI and SIK2 KI mice, homozygous SIK1/SIK2 KI mice were viable, fertile and indistinguishable from wild type. By contrast, homozygous SIK2/SIK3 KI was embryonically lethal, with lethality occurring after day E15.5 of gestation (Darling *et al.*, 2017).

Immunoprecipitation of each endogenous SIK isoform from tissue derived from the corresponding KI mice demonstrated that the kinase activity of each SIK isoform was completely ablated by the threonine to alanine mutation (Darling *et al.*, 2017). Subsequently, it was demonstrated that the phosphorylation of CRTC3 at serine 370 was substantially reduced in MEFs derived from SIK2/SIK3 double KI embryos compared with wild type MEFs (figure 3.4C-C), confirming that the kinase activity of SIK2 and SIK3 was abrogated in this cell line.

The TGFβ-mediated transcriptional induction of PAI-1 was then analysed between wild type and SIK2^{T175A}/SIK3^{T163A} MEF cell lines. Stimulation of wild type MEFs with recombinant TGFβ_1 for 6 hours resulted in the robust upregulation of PAI-1 protein expression (figure 3.4C-D). In contrast, the TGFβ-mediated upregulation of PAI-1 expression was substantially attenuated in SIK2^{T175A}/SIK3^{T163A} MEFs (figure 3.4C-D). This attenuation occurred despite the observation of elevated levels of receptor-mediated SMAD3 phosphorylation in the SIK2^{T175A}/SIK3^{T163A} MEFs compared with wild type control cells (figure 3.4C-D). Likewise, the attenuation of PAI-1 expression was also observed at the mRNA level. Stimulation of wild type MEFs with TGFβ resulted in a 40-fold increase in PAI-1 mRNA levels whereas the TGFβ-

mediated PAI-1 induction was significantly suppressed in two independent SIK2^{T175A}/SIK3^{T163A} MEF cell lines, with approximately 5-fold and 15-fold inductions in PAI-1 mRNA expression observed respectively (figure 3.4C-E).

This data therefore provides genetic evidence for the role of SIK isoforms in modulating the TGFβ-mediated transcriptional induction of the target gene PAI-1, supporting the observations detailed previously involving the small-molecule kinase inhibitor MRT199665 and siRNA-mediated SIK2/SIK3 depletion. However, there are a number of outstanding questions to be addressed in future research. Firstly, is the transcriptional upregulation of other TGFβ target genes in MEFs also influenced by the genetic inactivation of SIK2 and SIK3? Secondly, can the attenuation of PAI-1 transcription be rescued via the reintroduction of catalytically active SIK2 and SIK3 kinases? It would be also be interesting to further analyse individual SIK KI cell lines in order to ascertain whether all three isoforms contribute to the modulation of TGFβ transcriptional responses or whether it is specific to certain isoforms.

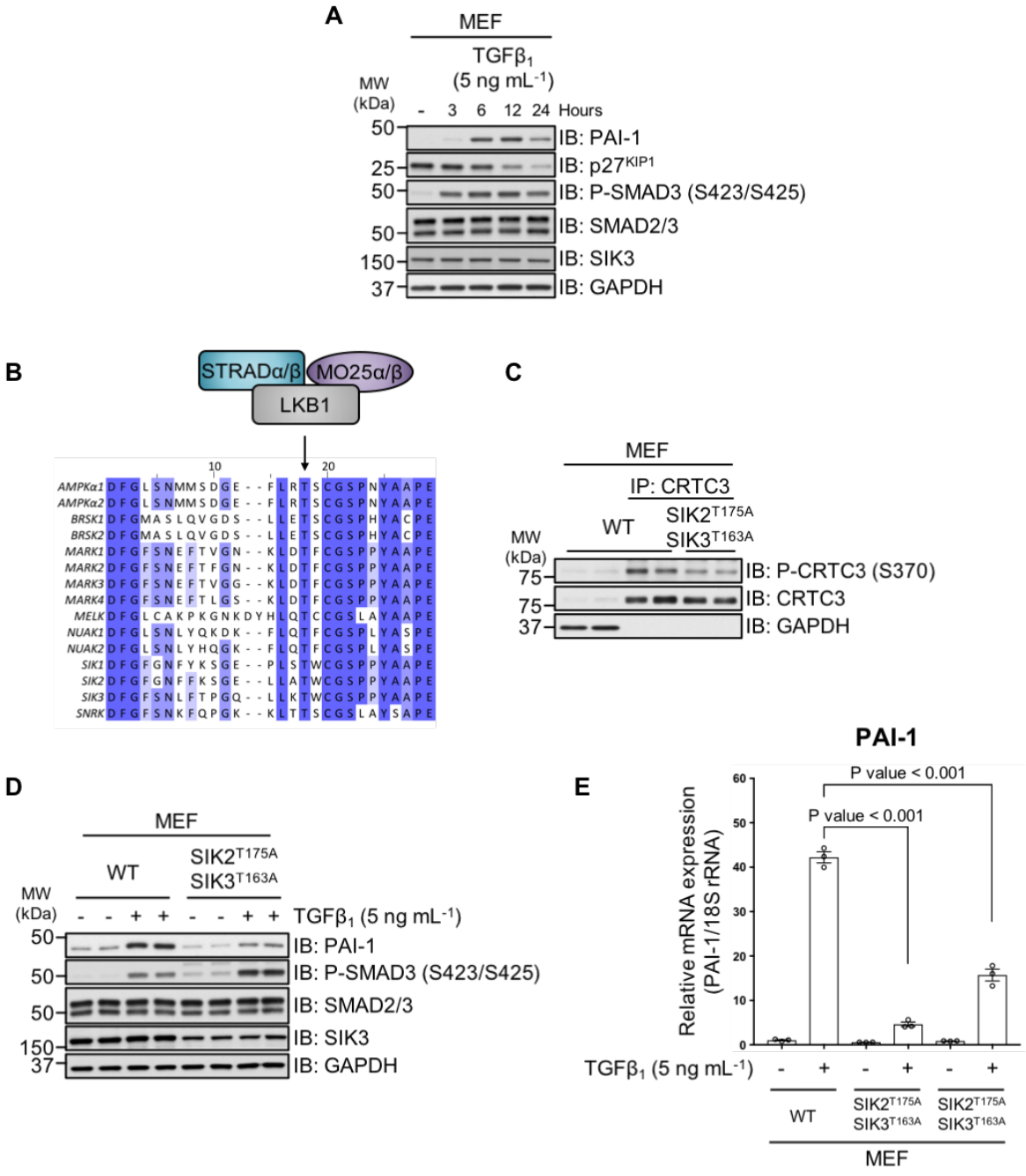


Figure 3.4C. The TGFβ-mediated transcriptional induction of PAI-1 is attenuated in MEFs derived from mouse embryos with genetically inactivated SIK2 and SIK3

(A) Wild type mouse embryonic fibroblasts (MEFs) were stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for the durations indicated prior to cell lysis. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently analysed via immunoblotting with the indicated antibodies. **(B)** The serine-threonine protein kinase LKB1, in complex with its regulatory subunits STRADα/β and MO25α/β, phosphorylates the invariant activation loop threonine residue of AMPK and 12 members of the AMPK-related family of protein kinases. Multiple protein sequence alignment was performed in Jalview (version 2.10.5) software (Waterhouse *et al.*, 2009) using the Clustal Omega sequence alignment programme. Highly conserved amino acid

residues are indicated by dark blue shading. **(C)** Cell lysate samples (800 µg total protein) obtained from either wild-type MEFs or SIK2^{T175A}/SIK3^{T163A} MEFs were subjected to immunoprecipitation of endogenous CRTC3 using anti-CRTC3 polyclonal sheep IgG conjugated to Protein G Agarose resin. Following elution from the antibody-resin, total cell lysate (20 µg protein) and IP samples were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently analysed via immunoblotting using the indicated antibodies. **(D)** Wild type and SIK2^{T175A}/SIK3^{T163A} MEFs were either unstimulated or stimulated with recombinant human TGFβ$_1$ (5 ng mL^{-1}) for 6 hours prior to cell lysis. Cell lysate samples (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently analysed via immunoblotting with the indicated antibodies. Immunoblot is representative of three independent experiments. **(E)** Wild type and two independent SIK2^{T175A}/SIK3^{T163A} MEF cell lines were either unstimulated or stimulated with recombinant TGFβ$_1$ (5 ng mL^{-1}) for 6 hours prior to lysis and RNA isolation. Complementary DNA (cDNA) was synthesised from the isolated RNA and RT-qPCR analysis performed for the indicated TGFβ target gene (*PAI-1*). Statistical analysis was performed on data obtained from three biological replicates.

3.4.5 Discussion

In the preceding sections 3.2 and 3.3, evidence for the involvement of SIK isoforms in regulating TGFβ-dependent transcriptional responses was presented involving the use of the small-molecule SIK inhibitor MRT199665. In this section, experiments involving alternative methods of disrupting SIK function have been presented that provide more robust evidence for the potential novel role of SIKs in TGFβ signalling.

Firstly, the TGFβ-induced transcription of the target gene PAI-1 was analysed in HeLa human cervical adenocarcinoma cells, which lack the endogenous expression of the serine-threonine protein kinase LKB1. LKB1 has been identified as the upstream activating kinase of SIK isoforms, along with AMPK and other members of the AMPK-related family (Lizcano *et al.*, 2004). In HeLa cells stably overexpressing wild type LKB1, both the basal and TGFβ-induced expression of PAI-1 mRNA and protein were significantly enhanced compared with the levels observed in normal HeLa cells. Importantly, the stable overexpression of a kinase inactive form of LKB1 (LKB1^{D194A}) failed to mediate the same effect. This therefore provides further evidence for the role of SIKs, however it does not exclude the involvement of protein kinases which as with the SIKs, are activated by LKB1 and inhibited by the MRT199665 compound, such as the four MARK isoforms (Lizcano *et al.*, 2004; Clark *et al.*, 2012).

Therefore, in order to further validate that the observed effects on TGFβ-mediated transcription were occurring through inhibition of the SIKs, experiments were performed in

which the protein levels were depletion via RNAi. An important point to state is that, in agreement with previous published research, I was unable to identify an antibody that could reliably immunodetect endogenous human or murine SIK1. Therefore, it was not possible to determine the efficacy of siRNA-mediated depletion of SIK1 protein expression via immunoblotting. In cells in which SIK2 and SIK3 protein levels were virtually undetectable by immunoblotting, the TGFβ-induced upregulation of PAI-1 protein expression appeared to be attenuated. However, in cells in which there was only a partial depletion of SIK protein expression, PAI-1 expression was comparable to the level observed in control cells. This indicated that the virtually complete depletion of SIK protein levels is required in order to observe the attenuation of TGFβ-induced PAI-1 expression. Despite this, the efficiency of siRNA-mediated protein depletion of SIK2 and SIK3 isoforms was inconsistent between independent experiments and thus an unreliable method by which to disrupt SIK function.

In order to investigate TGFβ-mediated transcription using a more robust experimental approach, we analysed the ability of TGFβ to induce PAI-1 expression in MEFs in which SIK2 and SIK3 isoforms had been genetically inactivated. In this context, the ability of TGFβ stimulation to induce the expression of PAI-1 of significantly impaired compared with control wild type MEFs. An important subsequent experiment to perform would be to express wild type SIK2 and SIK3 proteins in these cells and ascertain whether that is sufficient to restore the ability of TGFβ to upregulate PAI-1 expression. In addition, analysing TGFβ-mediated transcription in MEF cell lines in which the individual SIK isoforms have been genetically inactivated would facilitate in ascertaining whether the observed effects are mediated by specific SIK isoforms or whether there is redundancy.

3.5 THE CLINICALLY APPROVED SMALL-MOLECULE INHIBITORS BOSUTINIB AND DASATINIB INHIBIT SIK ISOFORMS AND REGULATE TGFβ SIGNALLING

3.5.1 Introduction

One of the principal recommendations when utilising small-molecule protein kinase inhibitors to interrogate cellular signalling pathways is to ensure that the effect observed occurs at a concentration similar to that which prevents the phosphorylation of a previously identified and validated physiological substrate of the protein kinase being investigated (Davies *et al.*, 2000; Cohen, 2009). Therefore, the small-molecule inhibitors utilised throughout this thesis to inhibit SIK isoforms were always tested in each different cell line employed to ensure they effectively prevented the phosphorylation of the *bona fide* SIK substrate CRTC3 (Clark *et al.*, 2012; MacKenzie *et al.*, 2013; Lombardi *et al.*, 2016b, 2017; Heap *et al.*, 2017) at the concentrations used. Furthermore, it is also important to ensure that the observed effect also occurs with at least two structurally unrelated chemical inhibitors (Davies *et al.*, 2000). As previously mentioned, the compounds HG-9-91-01 and MRT199665 are structurally distinct small-molecule inhibitors of SIK isoforms. However, unfortunately it was not possible to employ HG-9-91-01 throughout this thesis due to its off-target inhibition of the type I TGFβ receptor kinase. Interestingly, research has reported that the clinically approved small-molecule protein kinase inhibitors bosutinib and dasatinib are both capable of mediating the off-target inhibition of SIK isoforms (Sundberg *et al.*, 2014; Ozanne, Prescott and Clark, 2015) and therefore we decided to analyse whether both of these compounds exerted the same effect on TGFβ-dependent gene transcription as observed with MRT199665.

Bosutinib (Boschelli *et al.*, 2001; Golas *et al.*, 2003, 2005; Vultur *et al.*, 2008; Remsing Rix *et al.*, 2009) and dasatinib (Lombardo *et al.*, 2004; Das *et al.*, 2006) are both second-generation, ATP-competitive tyrosine kinase inhibitors (TKIs) (Weisberg *et al.*, 2007; Zhang, Yang and Gray, 2009). Bosutinib has been clinically approved by the US FDA (Food and Drug Administration) and EU European Medicines Agency (EMA) for the treatment of adult patients with chronic-phase Philadelphia chromosome-positive (Ph+) chronic myelogenous leukaemia (CML) that is resistant or intolerant to imatinib treatment (Cortes *et al.*, 2011; Trela, Glowacki and Błasiak, 2014; Rosti *et al.*, 2017). Dasatinib has also been clinically approved for the treatment of patients with imatinib-resistant or imatinib-intolerant chronic-phase Ph+ CML

in addition to Ph+ acute lymphoblastic leukaemia (ALL) resistant or intolerant to imatinib treatment (McFarland and Wetzstein, 2009; Kantarjian *et al.*, 2010; Keating, 2017; Rosti *et al.*, 2017).

The ability of both bosutinib and dasatinib to inhibit SIK isoforms was initially reported in a pharmacological screen aimed at identifying well-characterised small-molecule kinase inhibitors that could enhance the production of the anti-inflammatory interleukin-10 (IL-10) in murine bone-marrow-derived dendritic cells (BMDCs) (Sundberg *et al.*, 2014). Genetic perturbations that result in defective IL-10 signalling have been associated with the pathogenesis of inflammatory bowel disease (IBD) (Khor, Gardet and Xavier, 2011). IBD is a heterogenous group of idiopathic, chronic and relapsing inflammatory conditions of the gastrointestinal tract, the two most common forms of which are Crohn's disease and ulcerative colitis (UC) (Podolsky, 1991; Xavier and Podolsky, 2007). Genetic-linkage analysis and genome-wide association studies (GWAS) have identified mutations and single-nucleotide polymorphisms (SNPs) in genetic loci containing the *IL10* gene and the genes for its cognate receptor subunits *IL10RA* and *IL10RB* that are associated with an increased risk of developing Crohn's disease and UC (Glocker *et al.*, 2009; Jostins *et al.*, 2012). Clinical trials have demonstrated that administration of recombinant human IL-10 is safe and well-tolerated in patients. However, the majority of clinical studies to date have demonstrated only a non-significant clinical improvement following recombinant IL-10 treatment and have not observed any significant increase in the induction of clinical remission rates compared with placebo control treatments (Schreiber *et al.*, 2000; Marlow, van Gent and Ferguson, 2013). Therefore, research conducted by Sundberg *et al* (2014) aimed to identify FDA-approved small-molecule kinase inhibitors which enhance endogenous IL-10 production and therefore could be a potential therapeutic approach for the treatment of patients with IBD.

Bosutinib and dasatinib were amongst the most potent and active compounds that enhanced IL-10 production in BMDCs. However, the clinically approved TKI imatinib, which like bosutinib and dasatinib targets the ABL tyrosine kinase and is the first-line treatment for patients with Ph+ CML or ALL (Trela, Glowacki and Błasiak, 2014), was unable to upregulate IL-10 production. This suggested that the IL-10-potentiating effect of bosutinib and dasatinib were the result of shared off-target inhibition of other protein kinases. Subsequent analysis revealed that SIK isoforms (specifically SIK1 and SIK2) were enriched amongst the high-affinity targets of the IL-10-enhancing kinase inhibitors. Furthermore, treatment of BMDCs with

increasing concentrations of bosutinib resulted in a dose-dependent decrease in the phosphorylation of CRTC3 at the SIK-specific serine 370 (S370) residue. Importantly, short hairpin RNA (shRNA)-mediated depletion of endogenous CRTC3 protein expression abrogated the ability of both bosutinib and dasatinib to enhance IL-10 production in BMDCs. Collectively, the data from this research demonstrated that the clinically approved small-molecule kinase inhibitors bosutinib and dasatinib could enhance the production of the anti-inflammatory cytokine as a consequence of inhibiting SIK isoforms (Sundberg *et al.*, 2014).

	***In vitro* IC_{50} (nM)**		
TKI	**SIK1**	**SIK2**	**SIK3**
Bosutinib	< 3	< 3	18
Dasatinib	< 3	< 3	10
Erlotinib	-	1600	4000
Gefitinib	-	800	3800
Imatinib	-	> 100,000	> 100,000
Lapatinib	-	> 100,000	> 100,000
Sorafenib	-	> 100,000	> 100,000
Sunitinib	-	400	11,000
Vandetinib	370	120	740

Table 6. The *in vitro* IC_{50} values for nine clinically approved small-molecule tyrosine kinase inhibitors (TKIs) against the three SIK isoforms
The *in vitro* IC_{50} (nM) values were determined for nine small-molecule tyrosine kinase inhibitors (TKIs) against purified recombinant SIK1, SIK2 and SIK3 serine-threonine protein kinases. The data was obtained from (Ozanne, Prescott and Clark, 2015) and the *in vitro* IC_{50} determination was performed as described in (Hastie, McLauchlan and Cohen, 2006) by the inhibitor screening team at the MRC International Centre for Kinase Profiling (http://www.kinase-screen.mrc.ac.uk).

3.5.2 Bosutinib and dasatinib are potent inhibitors of SIK isoforms *in vitro* and in cultured cell lines

Bosutinib and dasatinib are both type 1 kinase inhibitors that interact with the highly conserved nucleotide-binding site of protein kinases through the formation of hydrogen bonds and hydrophobic interactions with residues in and around the region occupied by the adenine ring of ATP (Liu and Gray, 2006). Type 1 inhibitors constitute the majority of ATP-competitive kinase inhibitors and function by targeting the active conformation of the protein kinase in which the activation loop is phosphorylated (*i.e.* the conformation that is conducive to catalysing ATP phosphate transfer) (Liu and Gray, 2006; Zhang, Yang and Gray, 2009). By contrast, type 2 kinase inhibitors such as imatinib recognise the catalytically inactive conformation of the protein kinase in which the highly conserved tripeptide Asp-Phe-Gly (DFG) motif within the activation loop is folded away from the conformation required for ATP phosphotransfer (often referred to as the 'DFG-out' conformation) (Müller *et al.*, 2015). The DFG-out inactive conformation creates an additional hydrophobic pocket directly adjacent to the ATP binding site, frequently referred to as the allosteric binding site (Treiber and Shah, 2013). Consequently, type 2 kinase inhibitors interact with both the ATP binding site and form additional hydrophobic and hydrogen bonding interactions with residues in the allosteric binding site. It is proposed that type 2 inhibitors may exhibit higher kinase selectivity because the amino acid residues comprising the allosteric binding site are less conserved relative to those that form the ATP binding site (Liu and Gray, 2006). Thus, the ability of the activation loop of protein kinases to adopt distinctive inactive DFG-out conformations can facilitate the development of type 2 inhibitors with greater target selectivity (Schindler *et al.*, 2000).

In addition to inhibiting the constitutively active kinase domain of the BCR-ABL oncoprotein, bosutinib and dasatinib are potent inhibitors of multiple other protein tyrosine kinases including members of the Src family (Src, FGR, FYN, HCK, LCK, LYN and YES (Parsons and Parsons, 2004)), tyrosine-protein kinase BTK (also referred to as Bruton's tyrosine kinase), ephrin type-B receptor 4 (EPHB4), Mast/stem cell growth factor receptor KIT and platelet-derived growth factor receptor (PDGFR) (Das *et al.*, 2006; Bantscheff *et al.*, 2007; Weisberg *et al.*, 2007; Rosti *et al.*, 2017).

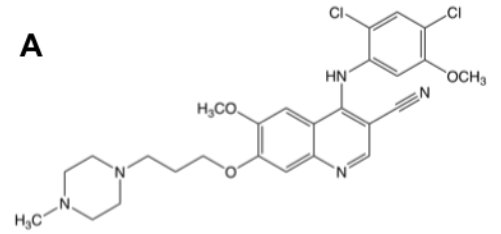
SIKs are unique amongst the AMPK-related family of serine-threonine protein kinases in that they possess a small threonine residue at the so termed 'gatekeeper position' at the periphery of the nucleotide-binding region within the catalytic domain. This is in contrast with

all the other members of the AMPK-related family which possess large hydrophobic gatekeeper residues. In the context of small-molecule kinase inhibitors, the importance of the gatekeeper residue is demonstrated by the observation that one of the most frequent point mutations identified in patients which develop imatinib-resistant CML involves mutation of the threonine 315 residue at the gatekeeper position (Quintás-Cardama, Kantarjian and Cortes, 2007; Weisberg *et al.*, 2007). The substitution of threonine 315 to a hydrophobic isoleucine residue (T315I) in the ABL catalytic domain abrogates an important hydrogen bond interaction between imatinib and BCR-ABL, resulting in imatinib resistance (Gorre *et al.*, 2001; Azam *et al.*, 2008). Therefore, it was hypothesised that various clinically-approved small-molecule TKIs including bosutinib and dasatinib may also be able to inhibit SIK isoforms due to the presence of the small threonine gatekeeper residue (Ozanne, Prescott and Clark, 2015). Consistent with the previously published research, kinase inhibitor profiling performed by the MRC International Centre for Kinase Profiling at the University of Dundee confirmed that both bosutinib and dasatinib are potent inhibitors of SIK isoforms *in vitro*, with comparable IC_{50} values to the clinically relevant target protein tyrosine kinases ABL, BTK and Src (figure 3.5A-B). Furthermore, bosutinib and dasatinib selectively inhibited SIK isoforms over other AMPK-related family members. Dasatinib in particular displays high selectivity towards SIK2 and SIK3 compared with all the other members of the AMPK-related family that were tested. Bosutinib appeared to be less selective than dasatinib, as it displayed inhibition of AMPK, MARK3 and NUAK1 protein kinases (table 7). However, it was substantially more potent towards SIK2 and SIK3. Importantly, neither bosutinib nor dasatinib exhibited any substantial inhibition of the type I TGFβ receptor kinase (TGFβR1) *in vitro* and were therefore considered suitable for use in this thesis.

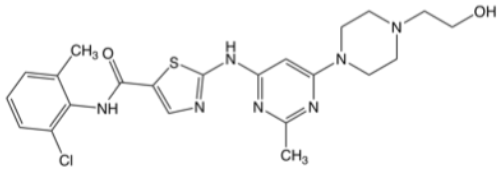
Therefore, it was subsequently tested whether bosutinib and dasatinib could effectively inhibit SIKs in cultured U2OS osteosarcoma cells. As a positive control, wild type U2OS cells were incubated with MRT199665 at 1 μM for 1 hour, which as expected inhibited the phosphorylation of the physiological SIK substrate CRTC3 at serine 370 (figure 3.5A-C). Incubation of U2OS cells with either bosutinib (3.0 μM) or dasatinib (0.3 μM) also resulted in a substantial reduction in CRTC3 serine 370 phosphorylation, with levels comparable to those observed with MRT199665 treatment (figure 3.5A-C). This data confirmed that both bosutinib and dasatinib were capable of inhibiting SIK isoforms under these experimental conditions

and in this cellular context and therefore we decided to investigate the effect of these two small-molecule kinase inhibitors on TGFβ signalling.

A



Bosutinib



Dasatinib

B

	Inhibitor IC_{50} values (nM)	
Protein Kinase	**Bosutinib**	**Dasatinib**
ABL	1.0	<1.0
BTK	15.0	1.0
Src	2.0	2.0
SIK1	<3.0	<3.0
SIK2	<3.0	<3.0
SIK3	18.0	10.0

C

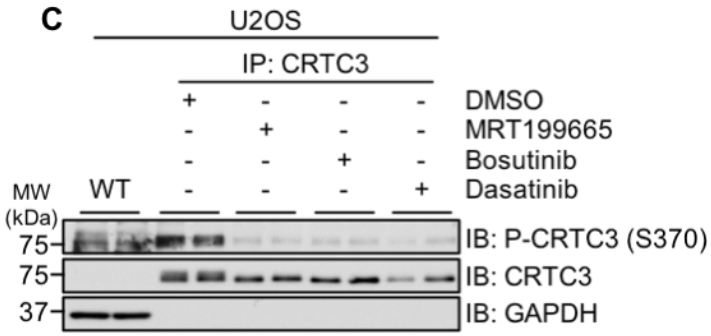


Figure 3.5A. Bosutinib and dasatinib are potent small-molecule inhibitors of SIK isoforms *in vitro* and cultured human U2OS osteosarcoma cells

(A) The chemical structures of the small-molecule tyrosine kinase inhibitors (TKIs) bosutinib and dasatinib. Molecular structures were created using ChemDraw Professional software (version 17.0) (PerkinElmer). **(B)** *In vitro* IC_{50} (nM) values for bosutinib and dasatinib against the clinically-relevant protein tyrosine kinases ABL, BTK and Src in addition to the three SIK serine-threonine protein kinases. The data was obtained from (Ozanne, Prescott and Clark, 2015) and the *in vitro* IC_{50} determination for bosutinib and dasatinib was performed by the inhibitor screening team at the MRC International Centre for Kinase Profiling (http://www.kinase-screen.mrc.ac.uk). **(C)** Wild type U2OS osteosarcoma cells were incubated with MRT199665 (1 µM), bosutinib (3.0 µM), dasatinib (0.3 µM) or an equivalent volume of DMSO for 1 hour prior to cell lysis. Cell lysate samples (800 µg total protein) were subjected to immunoprecipitation of endogenous CRTC3 using anti-CRTC3 polyclonal sheep IgG conjugated to Protein G Agarose resin. Following elution from the antibody-resin, total

cell lysate (20 µg protein) and IP samples were resolved via SDS-PAGE and subsequently transferred to nitrocellulose blotting membranes. The membranes were subjected to immunoblotting using the indicated antibodies.

3.5.3 Bosutinib and dasatinib both negatively regulate TGFβ-dependent gene transcription

As a result of the aforementioned research in which bosutinib and dasatinib were found to be potent small-molecule inhibitors of the SIK isoforms, it was decided to investigate whether they could recapitulate the effects of MRT199665 on TGFβ-dependent gene transcription. Similar to treatment with MRT199665, incubation of U2OS 2G transcriptional reporter cells with either bosutinib or dasatinib efficiently impaired the ability of TGFβ to induce the expression of GFP. Furthermore, treatment of wild type U2OS osteosarcoma cells with either bosutinib or dasatinib attenuated the TGFβ-dependent upregulation of the endogenous target gene *PAI-1*. As with MRT199665 treatment, the effect of bosutinib and dasatinib on TGFβ-dependent gene transcription appears to occur downstream of receptor-mediated phosphorylation of TGFβ-activated R-SMADs. Incubation of cells with either of the two small-molecule inhibitors did not substantially impact upon the phosphorylation of SMAD3 in response to TGFβ stimulation**.**

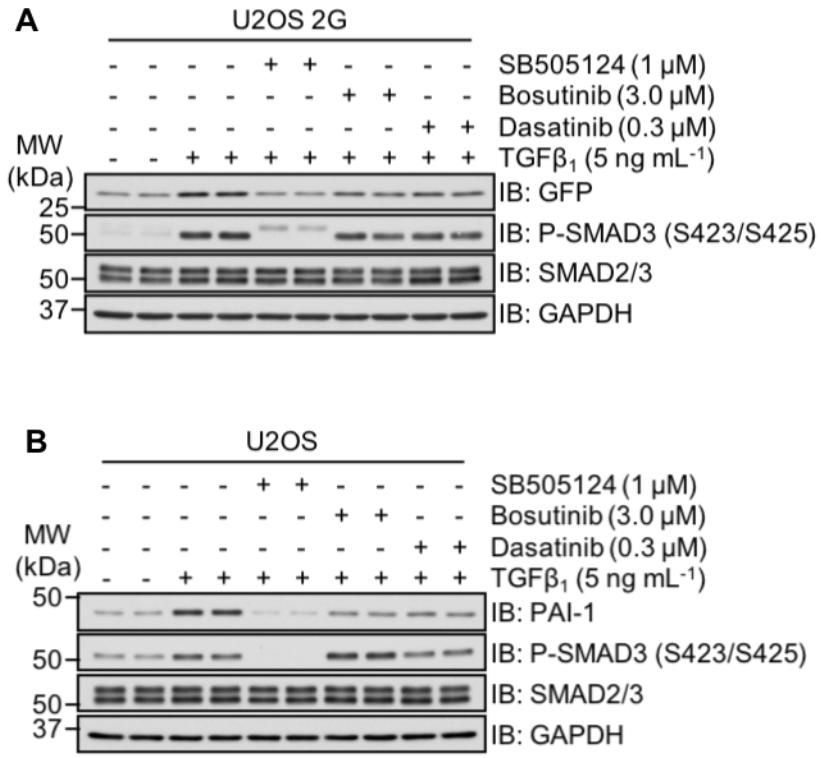


Figure 3.5B. The clinically approved small-molecule kinase inhibitors bosutinib and dasatinib both abrogate TGFβ-induced gene transcription in U2OS osteosarcoma cells
(A) U2OS 2G transcriptional reporter cells were incubated with SB-505124 (1 µM), bosutinib (3 µM), dasatinib (0.3 µM) or an equivalent volume of DMSO and stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 12 hours prior to cell lysis. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. **(B)** Wild type U2OS osteosarcoma cells were incubated with SB-505124 (1 µM), bosutinib (3 µM), dasatinib (0.3 µM) or an equivalent volume of DMSO and stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 6 hours prior to cell lysis. Cell lysates (12 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies.

3.5.4 Discussion

In this section, it has been demonstrated that the clinically approved small-molecule kinase inhibitors bosutinib and dasatinib are capable of inhibiting SIK isoforms in U2OS osteosarcoma cells. Furthermore, the inhibition of SIKs by bosutinib and dasatinib displays the same suppressive effect on TGFβ-dependent gene transcription as observed with the MRT199665 inhibitor and therefore this observation is significant for a number of factors.

Firstly, as mentioned previously, it is crucial that any effect observed occurs with at least two structurally unrelated inhibitor compounds (Davies *et al.*, 2000; Cohen, 2009). The HG-9-91-01 inhibitor is currently the most potent and selective inhibitor of SIK isoforms available and is structurally distinct compared with MRT199665. However, it was not suitable for use in this thesis due to the off-target inhibition of the type I TGFβ receptor kinase discussed in section 3.2. The caveat of only employing the use of a single small-molecule inhibitor is that any effect detected could conceivably occur due to inhibition of a protein kinase other than the kinase of interest due to inherent off-target effects of kinase inhibitors. Therefore, it was important identify alternative small-molecule inhibitors of SIKs with contrasting selectivity profiles and investigate whether they exerted the same effect on TGFβ signalling as MRT199665.

The data presented so far has demonstrated that three structurally unrelated small-molecule inhibitors (MRT199665, bosutinib and dasatinib) all display the same effect on TGFβ signalling. As observed with MRT199665, bosutinib and dasatinib both suppress the TGFβ-induced expression of GFP in the endogenous TGFβ transcriptional reporter cell line and also abrogate the upregulation of the endogenous TGFβ target gene PAI-1 in wild type osteosarcoma cells. One of the major limitations of the MRT199665 compound is that although it potently inhibits the SIK isoforms (SIK2 and SIK3 more than SIK1), it also targets a number of other members of the AMPK-related family in addition to AMPK itself. In particular, MRT199665 inhibits all four MARK isoforms and NUAK1 with *in vitro* IC_{50} values of 2-3 nM. Therefore, it would be problematic to draw any conclusions regarding which protein kinase is mediating the effects observed. However, because we also observed the same effects with both bosutinib and dasatinib, which exhibit different kinase selectivity profiles compared with MRT199665, it is therefore possible to eliminate the possibility of certain protein kinases. For example, although partial inhibition of the AMPK, MARK, MELK and NUAK1 kinases is observed with bosutinib *in vitro* (mean percentage activity remaining values in the range of 21-76% at 1 μM inhibitor concentration), it is much more potent towards SIK2 and SIK3 at the same concentration (mean percentage activity remaining values of 3% and 2% respectively). Furthermore, dasatinib displays no *in vitro* inhibition of any of the AMPK-related family protein kinases aside from SIK2 and SIK3 (mean percentage activity remaining values of 2%). An additional control experiment for the use of small-molecule TKIs in this context would be to treat cells with imatinib, which like bosutinib and dasatinib inhibits the protein tyrosine

kinases ABL and BTK however it does not inhibit SIK isoforms (Bantscheff *et al.*, 2007; Ozanne, Prescott and Clark, 2015). Therefore, one would predict that because of this it does not suppress the TGFβ-dependent upregulation of target gene transcription.

Therefore, the data presented in this section has demonstrated that the clinically approved small-molecule kinase inhibitors bosutinib and dasatinib exert the same suppressive effect as MRT199665 on the TGFβ-mediated induction of the target gene *PAI-1*. Although these three compounds have distinct kinase selectivity profiles, they are all potent inhibitors of SIK isoforms. Hence, it is likely that the effects observed on TGFβ signalling occurs through SIK inhibition. However, it is still important to verify that the attenuation of TGFβ-induced gene transcription is indeed mediated via the inhibition of SIK isoforms by using inhibitor-resistant SIK mutants.

	Bosutinib		**Dasatinib**	
Protein kinase	0.1 µM	1.0 µM	0.1 µM	1.0 µM
ABL	1 ± 0	0 ± 0	0 ± 0	0 ± 0
AMPK	65 ± 4	26 ± 2	107 ± 13	120 ± 5
BRSK1	101 ± 1	112 ± 5	126 ± 0	123 ± 1
BRSK2	107 ± 12	120 ± 4	102 ± 14	108 ± 13
BTK	1 ± 0	1 ± 0	1 ± 0	1 ± 0
LKB1	84 ± 13	104 ± 15	96 ± 18	100 ± 14
MARK1	84 ± 5	76 ± 4	115 ± 3	127 ± 3
MARK2	89 ± 8	68 ± 1	118 ± 4	126 ± 2
MARK3	74 ± 10	41 ± 3	96 ± 16	104 ± 5
MARK4	101 ± 7	72 ± 5	105 ± 12	115 ± 24
MELK	95 ± 10	66 ± 1	112 ± 7	128 ± 7
NUAK1	63 ± 12	21 ± 2	119 ± 10	118 ± 8
SIK2	8 ± 0	3 ± 0	4 ± 1	2 ± 1
SIK3	9 ± 0	2 ± 0	9 ± 2	2 ± 0
Src	1 ± 0	1 ± 0	1 ± 0	2 ± 0
TGFβR1	87 ± 14	101 ± 6	103 ± 1	82 ± 5

Table 7. Protein kinase profiling of the clinically approved small-molecule inhibitors bosutinib and dasatinib

The small-molecule inhibitors bosutinib and dasatinib were analysed for their ability to inhibit a panel of protein kinases *in vitro*. The panel included the clinically relevant non-receptor protein tyrosine kinases ABL, BTK and Src, in addition to the serine-threonine protein kinases LKB1, AMPK, AMPK-related family members and the type I TGFβ receptor (TGFβR1). The results indicate the mean percentage activity remaining ± standard deviation (SD). The data was adapted from (Ozanne, Prescott and Clark, 2015) and the *in vitro* protein kinase profiling was performed by the MRC PPU International Centre for Kinase Profiling at the University of Dundee (http://www.kinase-screen.mrc.ac.uk). Abbreviations: ABL, tyrosine-protein kinase ABL (also referred to as Abelson murine leukaemia viral oncogene homolog); AMPK, 5'-AMP-activated protein kinase; BRSK, Brain-specific serine/threonine-kinase; BTK, tyrosine-protein kinase BTK (also referred to as Bruton's tyrosine kinase); LKB1, serine/threonine protein kinase STK11 (also referred to as liver kinase B1); MARK, MAP/microtubule affinity-regulating kinase; MELK, Maternal embryonic leucine zipper kinase; NUAK1, NUAK family SNF1-like kinase 1; SIK, Salt-inducible kinase; Src, proto-oncogene tyrosine-protein kinase Src; TGFβR1, transforming growth factor-beta receptor type-1.

3.6 THE EFFECT OF SIK INHIBITION ON TGFβ-MEDIATED PHYSIOLOGICAL RESPONSES

3.6.1 Introduction

As alluded to previously, TGFβ is a multifunctional cytokine with the ability to regulate a diverse array of physiological responses, amongst the most prominent of which are cell proliferation, epithelial-mesenchymal transition (EMT), immune regulation and apoptotic cell death. From research conducted over the past two decades, it has become increasingly apparent that TGFβ signalling is context-dependent and a molecular framework involving the cooperation of SMADs with lineage-determining transcription factors (LDTFs), the cellular chromatin status and the input of additional signalling pathways has been proposed to explain the pleiotropic nature of TGFβ signalling (Massagué, 2012a; Ayyaz, Attisano and Wrana, 2017; David and Massagué, 2018). Further elucidation of how these contextual determinants define the cellular response to TGFβ signalling is of significant importance, particularly given the involvement of aberrant TGFβ signalling in the pathogenesis of multiple human diseases such as fibrosis and cancer progression and metastasis (Siegel and Massagué, 2003; Gordon and Blobe, 2008; Massagué, 2008a; Drabsch and ten Dijke, 2012).

The data outlined in the thesis thus far indicated a role for SIK isoforms in the modulation of TGFβ-mediated transcriptional regulation of certain target genes such as PAI-1, CTGF and SMAD7. Whilst this is a novel and interesting observation, it was important to also address the functional consequences of SIK activity on TGFβ-dependent cellular processes and therefore, the effect of MRT199665 on cellular proliferation and apoptosis was investigated.

3.6.2 MRT199665 enhances the upregulation of the cyclin-dependent kinase inhibitors $p21^{CIP1}$ and $p27^{KIP1}$

The ability of TGFβ to induce a cytostatic response in many different cell types has been extensively investigated primarily due to its major contribution to the tumour suppressive function of TGFβ signalling (Bierie and Moses, 2006; Massagué, 2008b; Inman, 2011b; Drabsch and ten Dijke, 2012). TGFβ-mediated cytostasis has been predominantly studied in

epithelial cells and principally occurs through two interconnected transcriptional responses (discussed in greater detail in section 1.4.4). Firstly, TGFβ can induce the expression of a number of cyclin-dependent kinase (CDK) inhibitors including $p15^{INK4B}$ (Hannon and Beach, 1994; Reynisdóttir *et al.*, 1995), $p21^{CIP1}$ (Datto, Yu and Wang, 1995; Reynisdóttir *et al.*, 1995) and $p27^{KIP1}$ (Polyak, Kato, *et al.*, 1994b; Polyak, Lee, *et al.*, 1994) which prevent progression through the G_1 phase of the cell cycle. Secondly, TGFβ also mediates the transcriptional repression of the cell growth-promoting transcription factor c-MYC which has been shown to be a critical aspect of the cytostatic response to TGFβ (Alexandrow and Moses, 1995; Alexandrow *et al.*, 1995; Chen, Kang and Massagué, 2001; Gomis *et al.*, 2006).

For the purpose of this thesis project, the cytostatic effect induced by TGFβ was analysed in two different epithelial cell types: HaCaT human immortalised keratinocyte cells and HuH-7 human hepatocellular carcinoma cells. Stimulation of HaCaT cells with recombinant human $TGFβ_1$ over a period of 48 hours rapidly suppressed the protein expression of the growth-promoting c-MYC transcription factor whilst concomitantly upregulated the expression of the CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (figure 3.6A-A). Similarly, stimulation of HuH-7 cells with recombinant $TGFβ_1$ over 48 hours also rapidly suppressed c-MYC protein expression and upregulated $p27^{KIP1}$ (figure 3.6A-A). The TGFβ-mediated cytostatic response appeared to be weaker in the HuH-7 cell line compared with the HaCaT cells, as the expression of $p27^{KIP1}$ did not increase until the 48-hour time point. In addition, unlike the expression observed in HaCaT cells, $p21^{CIP1}$ protein expression was not detected in HuH-7 cells. Thus, HaCaT cells appeared to have a more robust cytostatic response upon TGFβ stimulation and were therefore used in the subsequent experiments investigating cellular proliferation. The prolonged stimulation of HaCaT cells with recombinant TGFβ over a period of 7 days resulted in a significant suppression of cellular proliferation (figure 3.6A-B). The proliferation rates of unstimulated control HaCaT cells and those incubated with $TGFβ_1$ remained comparable until approximately day 5, at which point the unstimulated cells continued to proliferate whereas the proliferation rate of TGFβ-incubated cells was substantially reduced (figure 3.6A-B). This data confirmed that the HaCaT human keratinocyte cell line is a robust model in which to study TGFβ-induced cytostasis and were therefore used to analyse the effect of MRT199665 on this cellular response.

The stimulation of HaCaT cells with TGFβ resulted in a pronounced induction of the CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ protein expression, and the simultaneous repression of c-

MYC transcription (figure 3.6A-C). As expected, both of these TGFβ-mediated transcriptional responses were efficiently blocked in cells incubated with the TGFβ type I receptor kinase inhibitor SB-505124 (figure 3.6A-C). Interestingly, the incubation of cells with the small-molecule kinase inhibitor MRT199665 enhanced the protein expression of $p21^{CIP1}$ and $p27^{KIP1}$, and slightly reduced c-MYC protein expression even in the absence of TGFβ stimulation (figure 3.6A-C). The simultaneous incubation of cells with recombinant $TGF\beta_1$ and MRT199665 resulted in enhanced transcriptional induction of $p21^{CIP1}$ and $p27^{KIP1}$, and further repression of c-MYC compared with DMSO control TGFβ-stimulated cells (figure 3.6A-C). The effect of the MRT199665 inhibitor on the cellular proliferation of HaCaT cells was subsequently analysed, however the cells did not tolerate prolonged incubation with the compound and therefore the experiment was unable to be performed. As a result, it would be critical to determine whether bosutinib and dasatinib are capable of mediating the same effects as MRT199665 on CDK inhibitor and c-MYC expression in HaCaT cells. The effect of SIK inhibition on the cellular proliferation of HaCaT cells could then be analysed using either bosutinib or dasatinib. This data indicates that SIK inhibition by MRT199665 alone is sufficient to induce a cytostatic response in HaCaT cells, which is then augmented via the additional incubation of TGFβ.

Research has reported that TGFβ can concurrently induce apoptosis and EMT in cultured cells, which proposed the question of how a single cytokine can produce two distinct responses in the same cell type? Subsequent research demonstrated that the ability of TGFβ to induce either apoptosis or EMT is highly dependent on the cell cycle stage (Y. Yang *et al.*, 2006), uncovering an additional contextual determinant of TGFβ signal transduction, and suggested that TGFβ-induced growth arrest may function as a precondition for subsequent TGFβ-mediated cellular responses (Song, 2007). The data presented in this section indicates that there may also be a potential role for SIKs in modulating the cytostatic response in certain cell types and therefore in determining how cells respond to TGFβ stimulation.

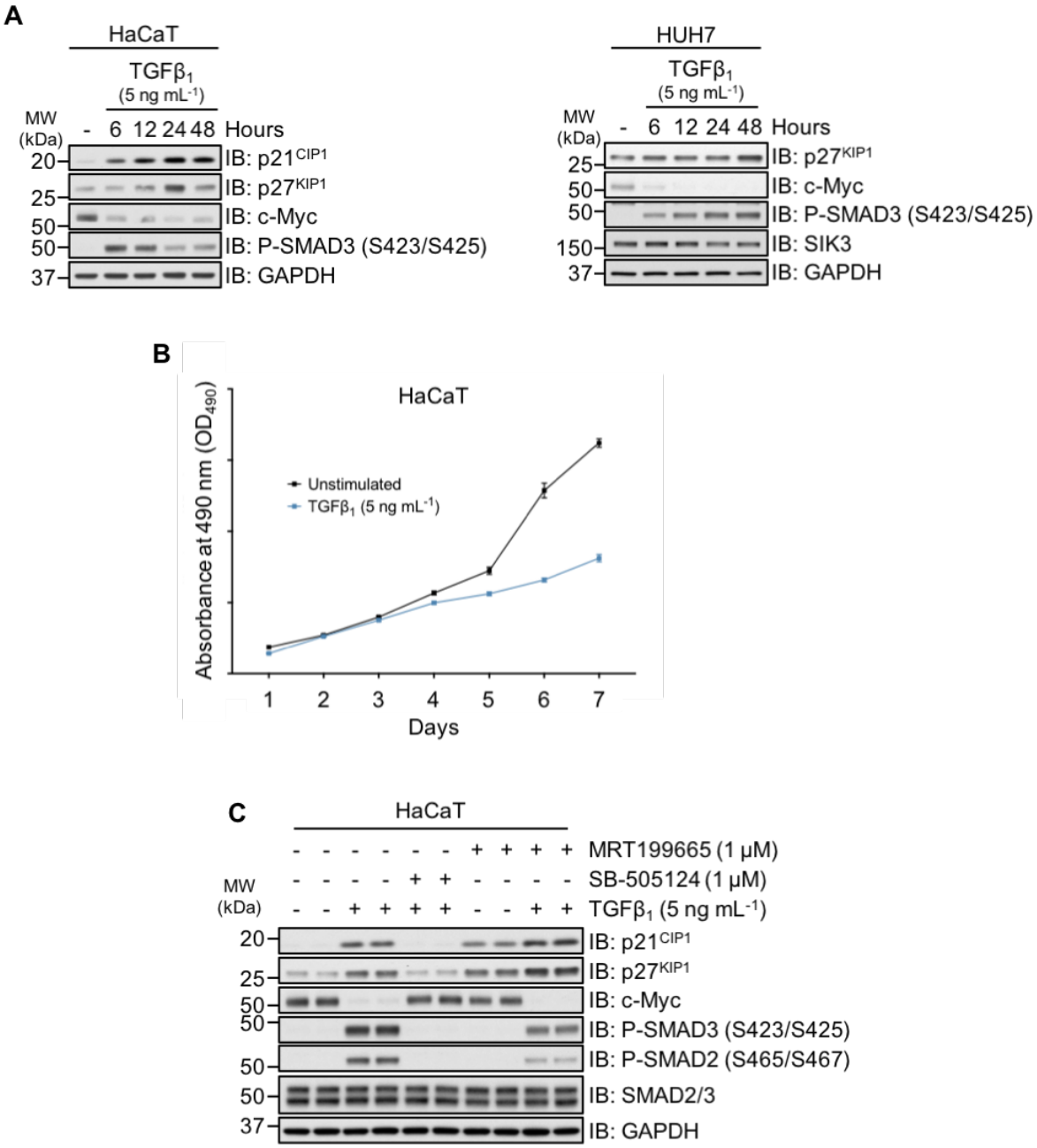


Figure 3.6A. MRT199665 enhances the cytostatic response in HaCaT human keratinocyte cells

(A) HaCaT human immortalised keratinocytes or HuH-7 human hepatocellular carcinoma cells were stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for the durations indicated prior to cell lysis. Cell lysates (10 µg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. **(B)** HaCaT keratinocyte cells were cultured in 96-well cell culture plates in the absence or presence of stimulation with recombinant human TGFβ_1 (5 ng mL^{-1}) for a period of 7 days. The cellular proliferation of a set of replicate wells was analysed every 24 hours. **(C)** HaCaT keratinocyte cells were incubated with SB-505124 (1 µM), MRT199665 (1 µM) or an equivalent volume of DMSO and stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 12 hours prior to cell lysis. Cell lysates (12 µg total protein) were

resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. The immunoblot is representative of two independent experiments.

3.6.3 NMuMG murine mammary epithelial cells undergo TGFβ-induced apoptosis

TGFβ signalling can either positively or negatively control the process of apoptosis in multiple different cell types and as with other physiological processes regulated by TGFβ, it appears to be highly dependent on cellular context (Schuster and Krieglstein, 2002; Zhang, Alexander and Wang, 2017). Although a coherent understanding regarding TGFβ-regulated apoptosis has yet to be established, a number of the proposed underlying molecular mechanisms are discussed in greater detail in section 1.4.5. For example, one of the principal mechanisms by which TGFβ can promote apoptosis appears to involve the SMAD-dependent transcriptional activation of BCL-2 protein family members (Ohgushi *et al.*, 2005; Ramjaun *et al.*, 2007; Spender *et al.*, 2009, 2013), critical modulators of the intrinsic (*i.e.* mitochondrial-dependent) apoptotic pathway (Youle and Strasser, 2008; Czabotar *et al.*, 2014).

The process of apoptosis can be investigated via a multitude of experimental techniques however one of the principal biochemical methods for analysing apoptotic cell death is the detection of proteins involved in mediating the apoptotic response (Banfalvi, 2017). The activation of caspase enzymes represents a fundamental event in the early apoptotic process (Nuñez *et al.*, 1998; Riedl and Shi, 2004; Li and Yuan, 2008; Taylor, Cullen and Martin, 2008; Shalini *et al.*, 2015) and therefore the detection of caspase activation is frequently employed to analyse apoptosis. In particular, the proteolytic activation of caspase-3, which along with caspase-6 and caspase-7 comprise the effector (alternatively termed executioner) caspases in mammals, is critical for the initiation of the mammalian apoptotic response (Fernandes-Alnemri, Litwack and Alnemri, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). The activation of caspase-3 requires the proteolytic processing of the inactive zymogen into large (p17) and small (p12) subunits. The two subunits associate with one another to form a caspase monomer, which then subsequently homodimerises via hydrophobic interactions with another monomer to form the active caspase enzyme (Riedl and Shi, 2004). The cleavage of caspase-3 occurs at the carboxy-terminal side of aspartic acid residue 175 (Asp175) and is mediated by the upstream initiator caspases caspase-8 and caspase-9 (Fernandes-Alnemri, Litwack and Alnemri, 1994; Tewari *et al.*, 1995; Shi, 2002;

Boatright and Salvesen, 2003). Therefore, the immunological detection of the large 17 kDa subunit of activated caspase-3 resulting from proteolytic cleavage following the Asp175 residue is one of the biochemical markers employed throughout this thesis in order to analyse apoptosis.

Upon proteolytic cleavage and activation, caspase-3 functions as an effector or 'executioner' caspase in the apoptotic programme and cleaves various substrates including the protein poly(ADP-ribose) polymerase (PARP) (Slee, Adrain and Martin, 2001; Elmore, 2007). PARP is a nuclear zinc finger DNA-binding protein that detects and binds to DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) and has important regulatory functions in cellular processes including DNA repair, chromosomal stability and apoptotic cell death (D'Amours *et al.*, 1999; Herceg and Wang, 2001). Once bound to the site of DNA strand breakage, PARP catalyses the transfer of the adenosine diphosphate (ADP)-ribose moiety from the substrate nicotinamide adenine dinucleotide (NAD^+) to various protein substrates involved in DNA repair (including PARP itself) and chromatin regulation(Oliver *et al.*, 1998), a post-translational modification termed reversible protein poly-ADP-ribosylation. The importance of PARP-mediated protein poly-ADP-ribosylation in the process of DNA repair has been further demonstrated by the observation that catalytic inhibitors of PARP have cytotoxic effects on BRCA1- or BRCA2-deficient cells and human tumours (Bryant *et al.*, 2005; Farmer *et al.*, 2005; Fong *et al.*, 2009). Breast cancer type 1 susceptibility protein (BRCA1) and breast cancer type 2 susceptibility protein (BRCA2; also referred to as Fanconi anaemia group D1 protein, FANCD1) are important for the repair of DNA double-strand breaks (DSBs) via homologous recombination (HR) and therefore critical for maintaining genomic stability (D'Andrea and Grompe, 2003; Narod and Foulkes, 2004; Gudmundsdottir and Ashworth, 2006; Roy, Chun and Powell, 2012). Consequently, BRCA1- and BRCA2-deficient cells are unable to efficiently repair DNA DSBs in response to various genotoxic insults and inhibition of PARP in these cells results in a high degree of genomic instability and ultimately cell death. Thus, multiple PARP inhibitors have been developed as a therapeutic strategy for the treatment of various human cancers and are currently undergoing clinical trials (Lord and Ashworth, 2008; Rouleau *et al.*, 2010).

The proteolytic cleavage of PARP has been identified as an early event to occur during the mammalian apoptotic response and one of the principal mediators of PARP cleavage *in vivo* is caspase-3 (Kaufmann *et al.*, 1993; Herceg and Wang, 2001; Slee, Adrain and Martin,

2001). PARP contains the caspase-3 tetrapeptide (P_4-P_3-P_2-P_1) recognition sequence Asp-Glu-Val-Asp (DEVD) (Thornberry *et al.*, 1997; Julien and Wells, 2017) and caspase-3 proteolytically cleaves the peptide bond between the Asp214 (at the P1 position) and Gly215 residues (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). PARP is comprised of three functional domains; an amino-terminal DNA-binding domain (DBD), a central auto-modification domain (AMD) and a carboxy-terminal catalytic domain (Oliver *et al.*, 1998; Rouleau *et al.*, 2010). Specific glutamate and lysine residues within the AMD function as acceptors for ADP-ribose moieties, thereby enabling PARP to poly-ADP-ribosylate (pADPr) itself (Altmeyer *et al.*, 2009; Tao, Gao and Liu, 2009). This auto-modification of PARP is proposed to have two major functions. Firstly, it may function as a scaffold and facilitate the recruitment of DNA repair proteins to sites of DNA damage and secondly, the large negative charge of the pADPr modification diminishes the affinity of PARP for DNA. Therefore, PARP dissociates from the site of DNA damage thereby providing access for the DNA repair proteins (Rouleau *et al.*, 2010). The caspase-3-mediated cleavage of PARP occurs within the DNA-binding domain and results in PARP inactivation. In the context of apoptosis, one of the proposed purposes for the cleavage and inactivation of PARP is to prevent unnecessary DNA repair from occurring during chromatin degradation.

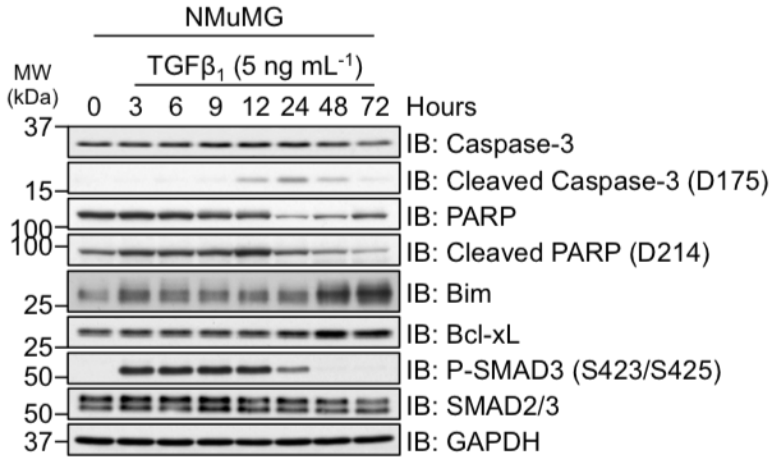


Figure 3.6B. TGFβ induces apoptotic cell death in NMuMG murine mammary epithelial cells
NMuMG murine mammary epithelial cells were incubated with recombinant human TGFβ_1 (5 ng mL^{-1}) for increasing durations. Both adherent and non-adherent cells (*i.e.* apoptotic cells) were lysed and cell lysates (12 μg total protein) were subsequently resolved via SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes which were subsequently subjected to immunoblotting with the indicated antibodies.

As previously discussed in section 1.4, signalling pathways induced by TGFβ contribute to numerous cellular processes including immunoregulation, EMT, cellular proliferation and apoptosis in a context-dependent manner. Therefore, we were interested to investigate whether the small-molecule kinase inhibitor MRT199665 exerted any effect on some of these processes.

TGFβ-mediated regulation of cellular processes such as apoptosis is highly dependent on the cellular context (Massagué, 2012b). For example, only certain cell types undergo apoptotic cell death in response to prolonged stimulation with recombinant $TGF\beta_1$ cytokine and therefore it is important to utilise the appropriate cell culture system. In order to investigate the effect of MRT199665 on the process of TGFβ-induced apoptosis, we employed the murine mammary epithelial cell line NMuMG, which have been extensively utilised for the interrogation of TGFβ-dependent apoptosis (Yu, Hébert and Zhang, 2002; Ramjaun *et al.*, 2007; Gal *et al.*, 2008; Viloria-Petit *et al.*, 2009; Avery-Cooper *et al.*, 2014; Liu *et al.*, 2017). The addition of recombinant human $TGF\beta_1$ ligand to the culture media of NMuMG cells caused extensive cell death and induced the proteolytic cleavage and hence activation of the effector caspase caspase-3. Immunodetection of the cleaved form of caspase-3 (D175) appeared following approximately 9 hours of TGFβ stimulation, with the highest levels observed at 24 hours. Concomitant with the cleavage and activation of caspase-3, $TGF\beta_1$ treatment of NMuMG cells also induces the cleavage (and hence inactivation) of the well-characterised substrate of caspase-3, PARP (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Protein levels of the full-length form of PARP (with an approximate molecular weight of 113 kDa) begin to decrease around 9 hour following 9 hours of $TGF\beta_1$ treatment and the lowest levels of full-length PARP occur after 24 hours of treatment. The caspase-3-mediated proteolytic cleavage of PARP occurs within the amino-terminal DNA-binding domain and results in the separation of the two zinc-finger DNA-binding motifs from the automodification domain and the carboxy-terminal pADPr catalytic domain, thereby impairing normal PARP function (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). PARP cleavage results in the formation of two polypeptides of approximately 89 kDa and 24 kDa molecular weight (Herceg and Wang, 2001). The appearance of the large 89 kDa fragment of cleaved PARP (D214) is maximal following approximately 12 hours of $TGF\beta_1$ stimulation. This data validates that the NMuMG murine mammary epithelial cell line is a

robust model for the investigation of TGFβ-mediated apoptosis in cell culture and in all subsequent experiments involving this cell line, cells were stimulated with recombinant TGFβ_1 for either 12 or 24 hours in order to induce the apoptotic cell death process.

3.6.4 Treatment of NMuMG cells with MRT199665 enhances the proteolytic cleavage of caspase-3 and PARP in response to TGFβ stimulation

Prior to investigating the effect of MRT199665 on TGFβ-mediated apoptosis, it was necessary to demonstrate that treatment of NMuMG cells with MRT199665 could effectively abrogate the phosphorylation of the SIK substrate CRTC3. Therefore, endogenous CRTC3 was immunoprecipitated from cells following treatment with either MRT199665, HG-9-91-01 or DMSO. As expected, both MRT199665 and HG-9-91-01 substantially reduced the phosphorylation of CRTC3 at serine 370 compared with DMSO control cells, indicating that both of these small-molecule inhibitors were effective at inhibiting SIK isoforms in NMuMG cells. Subsequently, we incubated with NMuMG cells with either DMSO, SB-505124 or MRT199665 in the presence of TGFβ_1 stimulation for 24 hours. In cells treated with DMSO, prolonged TGFβ stimulation inducted the process of apoptotic cell death, as indicated by the presence of detached cells in the culture media and the immunodetection of the proteolytically cleaved and activated form of caspase-3 in addition to the appearance of the large 89 kDa fragment resulting from PARP cleavage. Co-treatment of cells with the TGFβ type receptor kinase inhibitor SB-505124 completely prevented the apoptotic response from occurring. Interestingly, in cells co-incubated with MRT199665, the apoptotic response resulting from TGFβ1 stimulation appeared to be dramatically enhanced. Firstly, noticeably more detached (*i.e.* apoptotic) cells were observed in the culture media of cells incubated with MRT199665 compared with DMSO control treated cells when visualised via bright-field microscopy. Additionally, the immunodetection of the cleaved form of caspase-3 and the concomitant proteolytic cleavage of PARP were both substantially enhanced in cells incubated with MRT199665 in comparison to DMSO control cells. Consistent with previous experiments in different cell lines, MRT199665 exhibited no effect on the receptor-mediated phosphorylation of the TGFβ-activated R-SMADs SMAD2 or SMAD3, and SMAD protein expression remained consistent between all experimental conditions. Importantly, when NMuMG cells were incubated with MRT199665 without the addition of recombinant TGFβ_1

cytokine, neither the cleaved and activated form of caspase-3 or the large 89 kDa PARP fragment were detected. This indicated that treatment of cells with MRT199665 in isolation was not sufficient to induce apoptosis and that the observed potentiation of apoptotic cell death required both SIK inhibition and activation of the TGFβ signalling pathway.

In contrast to the attenuating effect of MRT199665 on the expression of the TGFβ target gene *PAI-1* in both U2OS human osteosarcoma and A-172 human glioblastoma cell lines, incubation of NMuMG cells with MRT199665 did not substantially impact the TGFβ-mediated induction of PAI-1 protein expression (figure 3.6C-D). It is conceivable that the as yet undetermined SIK substrate in the context of TGFβ signalling is not expressed in NMuMG cells and therefore incubation with the SIK inhibitor MRT199665 is unable to exert the same effect as observed in other cell lines. For example, this discrepancy may be the result of different SMAD transcriptional co-regulators being expressed in different cell types and further highlights the cell context-dependent nature of TGFβ signalling.

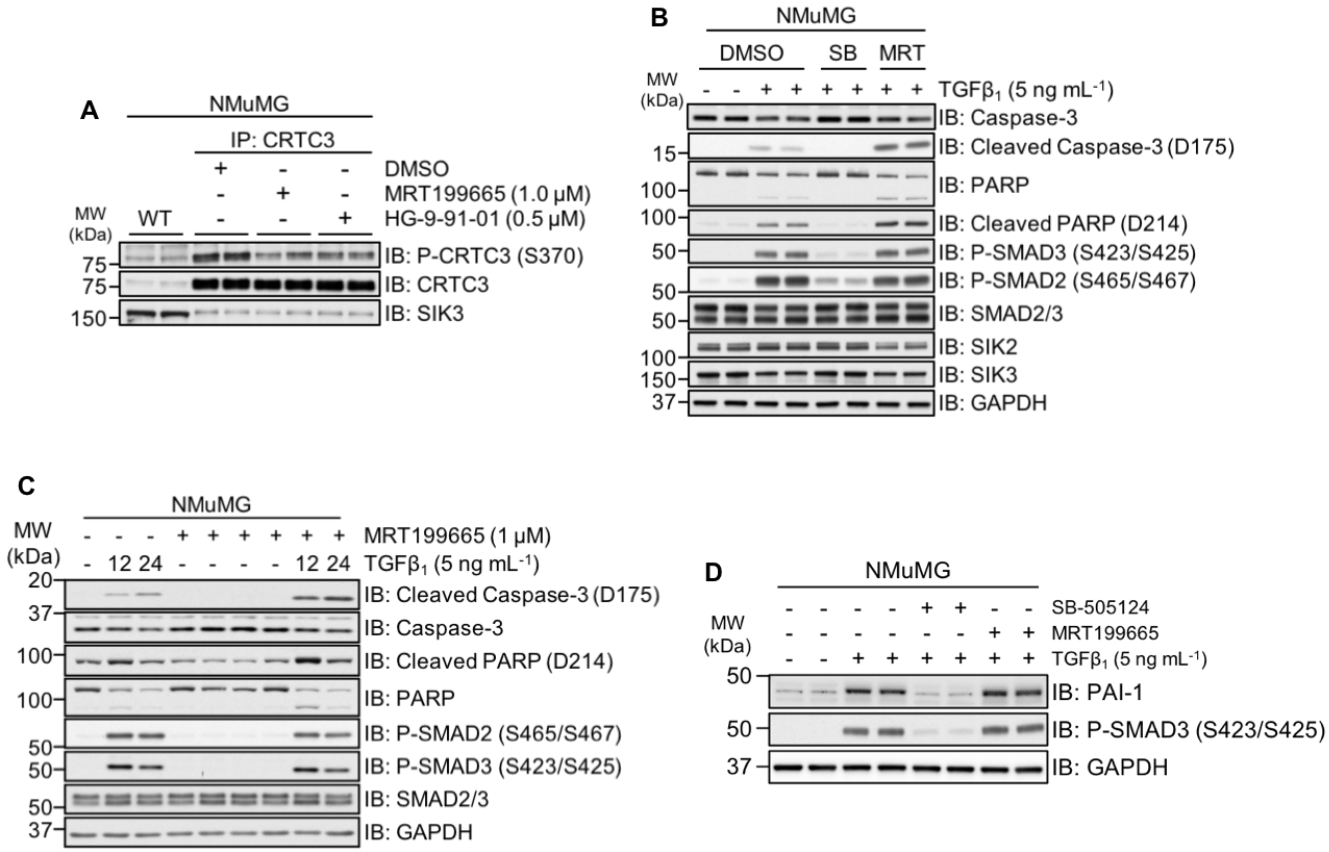


Figure 3.6C. Analysis of the small-molecule kinase inhibitor MRT199665 in the context of TGFβ-dependent apoptosis

(A) Wild type NMuMG murine mammary epithelial cells were incubated with MRT199665 (1 μM), HG-9-91-01 (0.5 μM) or an equivalent volume of DMSO for 1 hour prior to cell lysis. Cell lysate samples (600 μg total protein) were subjected to immunoprecipitation of endogenous CRTC3 using anti-CRTC3 polyclonal sheep IgG conjugated to Protein G Agarose resin. Following elution from the antibody-resin, total cell lysate (20 μg total protein) and IP samples were resolved via SDS-PAGE and subsequently transferred to nitrocellulose membranes. The membranes were subjected to immunoblotting using the indicated antibodies. **(B)** Wild type NMuMG murine mammary epithelial cells were incubated with SB-505124 (1 μM), MRT199665 (1 μM) or an equivalent volume of DMSO and stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 24 hours prior to cell lysis. Cell lysates (12 μg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies. **(C)** Wild type NMuMG murine mammary epithelial cells were incubated with either MRT199665 (1 μM) or an equivalent volume of DMSO, in the presence or absence of stimulation with recombinant human TGFβ_1 (5 ng mL^{-1}) for the indicated durations prior to cell lysis. Cell lysates (12 μg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting with the indicated antibodies.

3.6.5 Treatment of NMuMG cells with MRT199665 increases the percentage of apoptotic cells and reduces cellular viability

The various biochemical and morphological alterations that occur during the process of apoptosis can be exploited to discriminate between viable and non-viable (*i.e.* apoptotic) cells and can be utilised to determine the extent of apoptotic cell death that occurs under specific experimental conditions. Many of these cellular alterations including the activation of caspase enzymes, loss of mitochondrial membrane potential, changes in cellular DNA content and redistribution of plasma membrane phospholipids can be measured via flow cytometry (Vermes, Haanen and Reutelingsperger, 2000).

In normal viable cells, the distribution of various phospholipids is asymmetrical between the inner (*i.e.* intracellular side) leaflet and the outer (*i.e.* extracellular side) leaflet of the plasma membrane. The formation and modification of the plasma membrane phospholipid asymmetry is mediated by the concerted function of transmembrane lipid transporter enzymes P4-type ATPases, flippases, floppases and scramblases (van Meer *et al.*, 2006; van Meer, Voelker and Feigenson, 2008; Tanaka, Fujimura-Kamada and Yamamoto, 2011; Demchenko, 2013). Flippases and floppases belong to the ATP-binding cassette (ABC) transporter family and facilitate the ATP-dependent unidirectional translocation of

phospholipids between the outer and inner leaflets of the plasma membrane (van Meer *et al.*, 2006; Tanaka, Fujimura-Kamada and Yamamoto, 2011). P4-ATPases are a subfamily of the P-type ATPases that also mediate unidirectional phospholipid translocation in an ATP-dependent manner (Andersen *et al.*, 2016). By contrast, the scramblase enzymes catalyse the ATP-independent bidirectional translocation of phospholipids between the inner and outer leaflets of the plasma membrane. In this way, scramblases randomise the phospholipid distribution of the plasma membrane and therefore function to dissipate the phospholipid asymmetry (Hankins *et al.*, 2015; Montigny *et al.*, 2016). The dynamic regulation of the asymmetrical phospholipid composition of the plasma membrane appears to be critical for normal cellular function (Balasubramanian and Schroit, 2003).

The dissipation of the phospholipid asymmetry of the plasma membrane is a fundamental early event that occurs during the apoptotic process and involves the redistribution of the anionic aminophospholipid phosphatidylserine (PS) (Fadok *et al.*, 1992; Martin *et al.*, 1995; Erwig and Henson, 2008; Demchenko, 2013). In the majority of viable eukaryotic cell types, nearly all PS is sequestered in the inner leaflet of the plasma membrane (Ravichandran and Lorenz, 2007), however in cells undergoing apoptosis, PS is translocated to the outer plasma membrane leaflet (Fadok *et al.*, 1992; Martin *et al.*, 1995; Balasubramanian and Schroit, 2003). Research has indicated that PS translocation is facilitated by the activation of an ATP-independent non-specific phospholipid scramblase and the concomitant downregulation of an aminophospholipid-specific translocase (Bratton *et al.*, 1997; Fadok *et al.*, 1998; Segawa *et al.*, 2014; Montigny *et al.*, 2016).

The clearance of apoptotic cells is a fundamental process that occurs during development, injurious or pathological conditions and normal tissue homeostasis. It is critically important to rapidly remove apoptotic cells for the prevention of inflammation or autoimmune responses (Ravichandran and Lorenz, 2007; Erwig and Henson, 2008) and the principal function of PS translocation to the outer leaflet of the plasma membrane is to facilitate the recognition and effective engulfment of apoptotic cells via phagocytosis (Fadok *et al.*, 1992, 1998; Koopman *et al.*, 1994; Verhoven, Schlegel and Williamson, 1995). The exposure of PS on the apoptotic cell surface is recognised by phagocytic cells such as activated macrophages either directly through PS-recognition receptors (Miyanishi *et al.*, 2007; Park *et al.*, 2007, 2008; Das *et al.*, 2014) or indirectly via extracellular bridging molecules which

simultaneously interact with PS and membrane receptors on the macrophage cell surface (Ravichandran and Lorenz, 2007; Erwig and Henson, 2008).

The cell-surface exposure of anionic PS has been exploited in order to discriminate between healthy viable cells and those undergoing apoptotic cell death (Koopman *et al.*, 1994; Vermes *et al.*, 1995; van Engeland *et al.*, 1998; Vermes, Haanen and Reutelingsperger, 2000). Surface exposure of PS on apoptotic cells can be detected experimentally via the incubation of cell samples with annexin V conjugated to a fluorophore followed by flow cytometric analysis. Annexin V (also referred to as Annexin A5, placental anticoagulant protein-1 (PAP-1) or vascular anticoagulant alpha (VACα)) is a calcium-dependent phospholipid-binding protein which displays a high affinity for the anionic aminophospholipid PS (Tait, Gibson and Fujikawa, 1989; Andree *et al.*, 1990; Raynal and Pollard, 1994).

In the annexin V affinity staining assay, annexin V is unable to bind to normal healthy cells since the anionic PS is constrained to the inner plasma membrane leaflet and annexin is not able to penetrate the plasma membrane phospholipid bilayer. By contrast, in dead (*i.e.* necrotic) cells, the integrity of the plasma membrane is compromised and consequently annexin V can bind to PS on both the inner and outer plasma membrane leaflet (van Engeland *et al.*, 1998). The extracellular exposure of PS occurs during the early phase of the apoptotic process in which the integrity of the plasma membrane remains intact. Therefore, in order to discriminate between viable cells, early apoptotic cells and necrotic cells, the assay involves the simultaneous addition of a plasma membrane impermeable DNA stain to the cell suspension samples. Hence, when the cell suspension samples are analysed via flow cytometry, intact viable cells remain unstained, early apoptotic cells (retaining plasma membrane integrity) are stained by the annexin V fluorophore conjugate, whereas necrotic cells are dual stained by the annexin V fluorophore conjugate and the DNA stain (Vermes *et al.*, 1995; van Engeland *et al.*, 1998; Vermes, Haanen and Reutelingsperger, 2000). During the experimental procedure, the cells are not subject to permeabilisation or fixation and therefore only the cells in the later stages of the apoptotic process in which the plasma membrane begins to disintegrate and become permeable will be stained with DAPI.

The treatment of NMuMG cells with recombinant human TGFβ_1 for 12 hours enhanced the appearance of annexin V positive cells, demonstrating an increase in the percentage of cells undergoing TGFβ-mediated apoptosis (figure 3.6D-A). Annexin V staining, and by extension the apoptotic response, was significantly reduced in cells co-incubated with

the TGFβ type I receptor kinase inhibitor SB-505124. However, when cells were incubated simultaneously with both TGFβ_1 and the small-molecule kinase inhibitor MRT199665, the percentage of cells stained with annexin V was significantly enhanced compared to DMSO control TGFβ stimulated cells (figure 3.6D-A). This indicates that the MRT199665 inhibitor is able to potentiate the apoptotic response in response to TGFβ stimulation. Furthermore, in the same experimental conditions, the percentage of viable intact cells, as determined by those cells which are not amenable to DAPI staining, is significantly decreased in cells treated simultaneously with MRT199665 and recombinant TGFβ_1, compared with TGFβ-treated DMSO control cells (figure 3.6D-B).

In order to compliment the aforementioned annexin V staining assay, the cellular viability of NMuMG cells under various experimental conditions was also analysed using the crystal violet staining method. This is based on the observation that when adherent cells in culture undergo apoptotic cell death, they become detached from the cell culture plate. The adherent cells can be stained using the crystal violet dye which binds to proteins and DNA. The apoptotic cells which have lost their adherence are removed from the cell population, thereby reducing the amount of crystal violet staining that is subsequently detected (Feoktistova, Geserick and Leverkus, 2016). When NMuMG cells were incubated with recombinant human TGFβ_1 for 24 hours, the relative cellular viability decreased by approximately 20 percent compared to unstimulated DMSO control cells (figure 3.6D-C). The co-treatment of cells with the SB-505124 inhibitor in addition to TGFβ stimulation prevented the reduction in cell viability. By contrast, when cells with simultaneously incubated with the MRT199665 small-molecule inhibitor and recombinant TGFβ_1, the relative cellular viability was significantly attenuated compared to DMSO control cells stimulated with TGFβ (figure 3.6D-C). Collectively, the data presented in this section has demonstrated through a number of experimental techniques that the small-molecule kinase inhibitor MRT199665 can potentiate the TGFβ-mediated apoptotic response in NMuMG cells. In subsequent research, it would be important to conduct similar annexin V and crystal violet staining assays in order to assess whether the two other small-molecule kinase inhibitors of SIK isoforms used in this thesis project, bosutinib and dasatinib, are also capable of potentiating TGFβ-induced apoptotic cell death in NMuMG cells. Moreover, it would also be imperative to analyse SIK inhibition in the context of alternative cell lines that are amenable to TGFβ-mediated

apoptotic cell death such as the murine hepatocyte cell line AML-12 which has been previously used to investigate the process of TGFβ-mediated apoptosis (Ramjaun *et al.*, 2007).

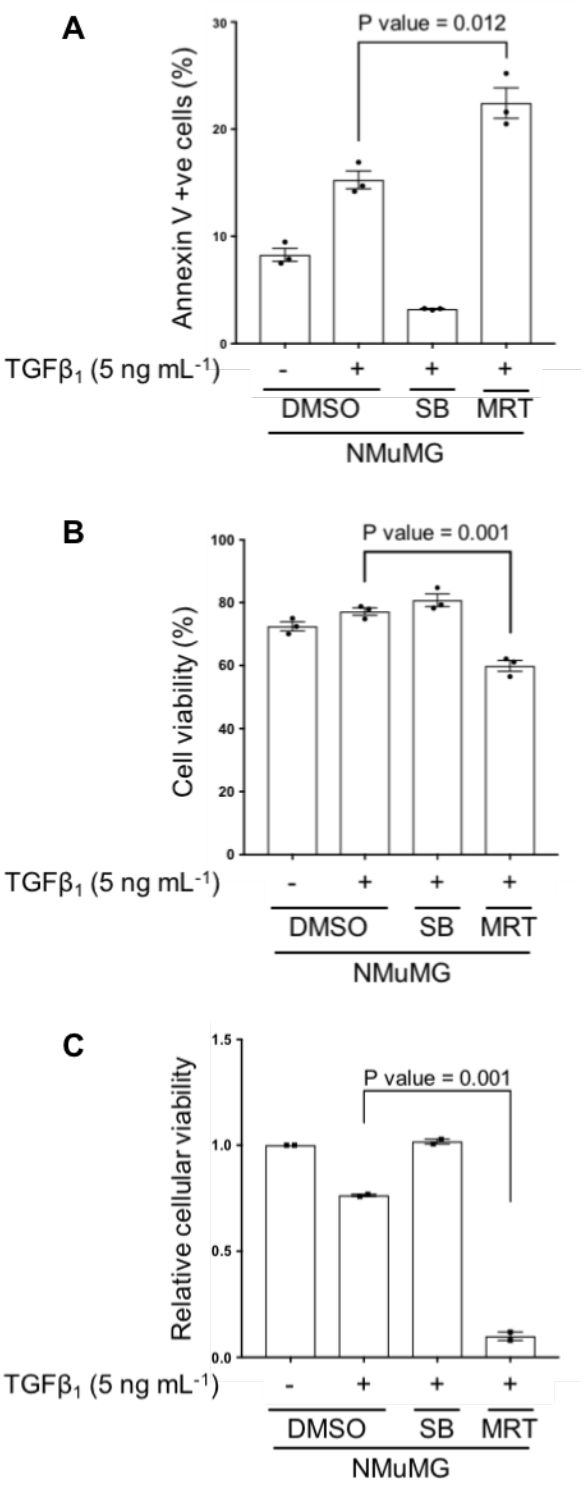

A
P value = 0.012
Annexin V +ve cells (%)
30
20
10
0
TGFβ1 (5 ng mL-1)
-
+
+
+
DMSO
SB
MRT
NMuMG
B
P value = 0.001
Cell viability (%)
100
80
60
40
20
0
TGFβ1 (5 ng mL-1)
-
+
+
+
DMSO
SB
MRT
NMuMG
C
P value = 0.001
Relative cellular viability
1.5
1.0
0.5
0.0
TGFβ1 (5 ng mL-1)
-
+
+
+
DMSO
SB
MRT
NMuMG

Figure 3.6D. The small-molecule kinase inhibitor MRT199665 potentiates TGFβ-mediated apoptotic cell death in NMuMG cells
(A) NMuMG murine mammary epithelial cells were incubated with SB-505124 (1 μM), MRT199665 (1 μM) or an equivalent volume of DMSO and simultaneously stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 12 hours. Adherent and non-adherent (*i.e.* apoptotic) cells were collected and prepared for flow cytometric analysis using dual-staining (Annexin V/DAPI) as detailed in section 2.2.17. **(B)** NMuMG murine mammary epithelial cells were incubated with SB-505124 (1 μM), MRT199665 (1 μM) or an equivalent volume of DMSO and simultaneously stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 12 hours. Adherent and non-adherent (*i.e.* apoptotic) cells were collected and prepared for flow cytometric analysis using dual-staining (Annexin V/DAPI) as detailed in section 2.2.17. **(C)** NMuMG murine mammary epithelial cells were seeded in 96-well cell culture plates and incubated with SB-505124 (1 μM), MRT199665 (1 μM) or an equivalent volume of DMSO and simultaneously stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 24 hours. Cells were subjected to methanol fixation and cellular viability was analysed via crystal violet staining assay as detailed in section 2.2.18.

3.6.6 Treatment of NMuMG cells with bosutinib enhances the proteolytic cleavage of caspase-3 and PARP in response to TGFβ stimulation

As discussed in section 3.5, the clinically approved small-molecule tyrosine kinase inhibitors (TKIs) bosutinib and dasatinib have been shown to inhibit SIK isoforms and both of these inhibitors exert the same effect as MRT199665 on TGFβ-dependent transcriptional regulation of the target gene PAI-1. As previously discussed, it is important to demonstrate that any effects observed occur with multiple structurally unrelated small-molecule inhibitors and therefore it was important to investigate whether bosutinib or dasatinib could also potentiate TGFβ-mediated apoptosis. Firstly, we wanted to demonstrate that neither bosutinib nor dasatinib affected the receptor-mediated phosphorylation of the TGFβ-activated R-SMAD SMAD3 in the NMuMG murine mammary epithelial cell model employed for the study of TGFβ-induced apoptosis. In NMuMG cells incubated with either bosutinib or dasatinib, SMAD3 phosphorylation levels were comparable with the levels observed in DMSO control treated cells. Subsequently, we analysed whether bosutinib could recapitulate the potentiation of TGFβ-mediated apoptosis that was observed when cells were co-incubated with MRT199665. Indeed, in cells co-incubated with bosutinib and recombinant TGFβ_1 for 24 hours, we detected enhanced levels of cleaved (hence activated) caspase-3 and resulting cleavage of the caspase-3 substrate PARP, compared to levels observed in DMSO control treated cells. Although the levels of cleaved caspase-3 and cleaved PARP were less than those

observed with MRT199665 treatment, this provides evidence that two structurally unrelated small-molecule kinase inhibitors of SIK isoforms exert a similar effect on the TGFβ-mediated apoptotic cell death of NMuMG cells. Subsequent experiments using alternative methods of detecting the process of apoptosis (such as annexin V staining and crystal violet staining employed previously in this thesis or DNA-fragmentation assay) are required in order to validate the effect of bosutinib on TGFβ-mediated apoptosis. Furthermore, it would be advantageous to investigate whether dasatinib is also capable of exerting similar effects as MRT199665 and bosutinib.

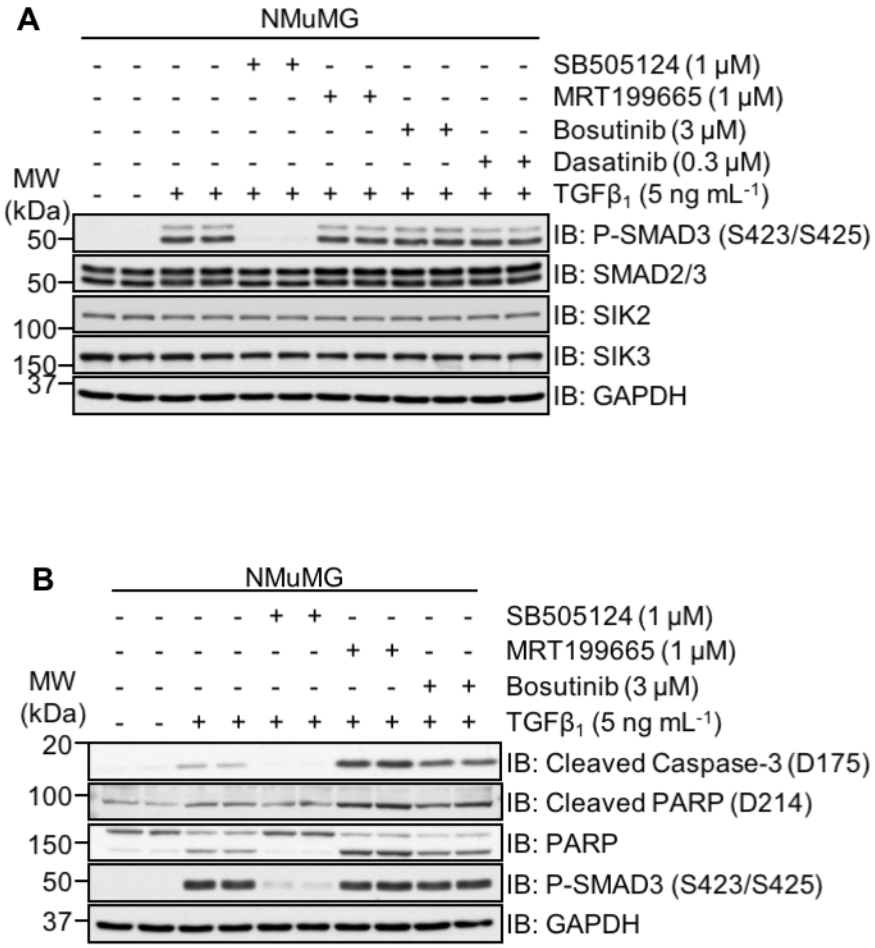


Figure 3.6E. The small-molecule TKI bosutinib potentiates the TGFβ-mediated apoptotic response in NMuMG cells

(A) Wild type NMuMG murine mammary epithelial cells were incubated with the small-molecule kinase inhibitors SB-505124 (1 μ), MRT199665 (1 μM), bosutinib (3 μM) or dasatinib (0.3 μM), or an equivalent volume of DMSO and simultaneously stimulated with recombinant human TGFβ_1 (5 ng mL^{-1}) for 1 hour prior to cell lysis. Cell lysates (12 μg total protein) were resolved via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes

were subsequently subjected to immunoblotting using the indicated antibodies. **(B)** Wild type NMuMG murine mammary epithelial cells were incubated with SB-505124 (1 μM), MRT199665 (1 μM), bosutinib (3 μM) or an equivalent volume of DMSO and simultaneously stimulated with recombinant human TGFβ1 (5 ng mL-1) for 24 hours prior to cell lysis. Cell lysate samples (12 μg total protein) were separated via SDS-PAGE and transferred to nitrocellulose blotting membranes. The membranes were subsequently subjected to immunoblotting using the indicated antibodies. The immunoblot is representative of two independent experiments.

3.6.7 Discussion

In this section, it has been demonstrated that inhibition of SIK isoforms with the structurally unrelated small-molecule kinase inhibitors MRT199665 and bosutinib can potentiate the TGFβ-mediated apoptosis of NMuMG cells in culture. Although this is an interesting and novel observation, there remains a number of outstanding crucial questions that need to be addressed in future experiments. Firstly, despite observing this enhancement of TGFβ-mediated apoptosis using two distinct small-molecule inhibitors of SIK isoforms (with contrasting kinase selectivity profiles), it is still conceivable that the effect observed is occurring due to inhibition of an 'off-target' protein kinase. This can be somewhat addressed by performing the same experiments as presented above but using dasatinib, another compound known to inhibit SIKs that is structurally unrelated to either MRT199665 or bosutinib. If treatment of NMuMG cells with dasatinib substantiates the effect on TGFβ-mediated apoptosis, it would provide additional evidence that the effect is occurring through inhibition of SIK isoforms. Furthermore, it would also be interesting to investigate the effect of imatinib treatment in this context. Imatinib, similar to bosutinib and dasatinib, is a clinically approved small-molecule TKI which inhibits a number of the protein kinases that bosutinib and dasatinib also target. Importantly however, it is not capable of inhibiting any of the three SIK isoforms and therefore one would postulate that it is unable to potentiate the TGFβ-mediated apoptotic cell death of NMuMG cells. Again, this would provide further evidence that this effect is occurring through SIK kinase inhibition.

In this thesis, the ability of MRT199665 and bosutinib to potentiate apoptosis mediated via TGFβ has only been demonstrated in NMuMG normal murine mammary epithelial cells. However, there are numerous other cell lines that have been utilised by researchers in order to investigate this process including the AML12 normal murine

hepatocyte cell line and multiple Burkitt's lymphoma (BL) cell lines such as Ramos, BL2, BL40 and BL41 (Chaouchi *et al.*, 1995; Saltzman *et al.*, 1998; Schrantz *et al.*, 1999; Inman and Allday, 2000b, 2000a; Ramjaun *et al.*, 2007; Spender *et al.*, 2009, 2013). It would be very important and interesting to resolve these problems in order to investigate whether MRT199665 or bosutinib are capable of potentiating the TGFβ-mediated apoptotic cell death of these cell lines as observed in NMuMG cells.

If subsequent research provides robust evidence that the attenuation of SIK catalytic activity using small-molecule kinase inhibitors can potentiate TGFβ-mediated apoptosis of multiple human cancer cell lines in culture, this would generate interesting therapeutic possibilities. The ability of TGFβ to function as both a tumour suppressor or tumour promoter depending on the cellular context was previously discussed in detail in section 1.5. In brief, TGFβ exerts a tumour suppressive effect in normal premalignant cells however perturbations in the signalling pathway, either through inactivation of core components or defects in downstream signalling responses, can enable cells to evade the tumour suppressive action of TGFβ. Moreover, malignant cells can corrupt TGFβ signalling via mechanisms such as induction of EMT and angiogenesis in order to promote cancer progression and metastasis. Previous research has demonstrated that TGFβ can induce apoptotic cell death in multiple different human B-cell lymphoma cell lines (Chaouchi *et al.*, 1995; Saltzman *et al.*, 1998; Schrantz *et al.*, 1999; Inman and Allday, 2000b, 2000a, Spender *et al.*, 2009, 2013). Thus, the ability of small-molecule inhibitors of SIKs to potentiate TGFβ-mediated apoptosis may represent a novel potential therapeutic approach to the targeted treatment of specific forms of lymphoma and for this reason, it would be of immense interest to investigate the ability of SIK inhibitors to augment TGFβ-dependent apoptosis in an *in vivo* murine model. Understandably, there would be numerous additional factors to consider for this potential approach to be efficacious. For example, it would only specifically be of therapeutic value for tumour cells that are responsive to TGFβ signalling and furthermore, tumour cells that are amenable to TGFβ-induced apoptosis. Additionally, there may be a requirement for administration of recombinant human $TGF\beta_1$ if the circulating plasma concentration is insufficient to induce apoptotic cell death. The viability and safety of this would require extensive research, particularly with the knowledge that TGFβ can function as a tumour promoter in certain cellular contexts. However, the observation that bosutinib and dasatinib

are both potent inhibitors of SIK isoforms suggests that specific and potent small-molecule SIK inhibitors will be tolerated in humans.

Recent research has sought to optimise the small-molecule SIK inhibitor HG-9-91-01 to develop analogs that have improved protein kinase selectivity and that are suitable for use *in vivo* (Sundberg *et al.*, 2016). Although HG-9-91-01 is a potent inhibitor of SIK isoforms, it is known to mediate the 'off-target' inhibition of other kinases, including the TGFβ type I receptor serine-threonine kinase as demonstrated in this thesis project. Furthermore, it exhibits poor pharmacokinetic (PK) properties, with a high degree of plasma protein binding and rapid degradation rendering it unsuitable for *in vivo* use. The research conducted by Sundberg *et al* reported the development of a novel SIK inhibitor YKL-05-099 that displays an increased selectivity for SIK isoforms versus other protein kinases and improved PK properties that enable it to be used for in vivo study. However, despite the improvements to the kinase selectivity profile, I have analysed the effect of YKL-05-099 on TGFβ signalling and observed that it attenuates the receptor-mediated phosphorylation of SMAD3, most likely as a result of inhibition of the TGFβ type I receptor kinase as observed with HG-9-91-01. Therefore, neither HG-9-91-01 or YKL-05-099 are suitable for the *in vivo* investigation of SIK inhibition in the context of TGFβ signalling regulation.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the main discovery of this doctoral project has been the identification of salt-inducible kinases (SIKs) as novel protein kinase regulators of the TGFβ signalling pathway. The data presented within this thesis provides evidence for the involvement of SIKs in modulating specific transcriptional responses and cellular functions downstream of TGFβ pathway activation. Perturbation of the catalytic activity of SIKs, either by the use of small-molecule kinase inhibitors or via genetic inactivation, attenuates the TGFβ-mediated transcriptional induction of certain target genes. Moreover, this modulation of TGFβ-dependent transcriptional responses does not involve the abrogation of receptor-mediated phosphorylation of SMAD transcription factors nor the disruption of SMAD nuclear translocation in response to TGFβ stimulation.

The research conducted for this thesis project has demonstrated that the endogenous 2G TGFβ-dependent transcriptional reporter cell line previously developed via collaboration between the Hay and Sapkota research groups (Rojas-Fernandez *et al.*, 2015) is a valuable research tool and can be effectively exploited for high-throughput screening (HTS) methods. Following on from the validation that the endogenous transcriptional reporter cell line is a robust system in which to perform HTS, it would be interesting in future research to perform pharmacological screening using more extensive small-molecule inhibitor libraries. This would allow for greater coverage of the human kinome and to pharmacologically target other classes of enzymes involved in cellular signalling such as E3 ubiquitin-protein ligases and DUBs. Additionally, due to dual-reporter nature of the cell line, it would be beneficial to perform dual-screening using both GFP fluorescence and luciferase enzyme activity in order to improve the robustness and reliability of the data.

The methodology employed to generate the endogenous 2G TGFβ-dependent transcriptional reporter cell lines can also be used to create endogenous transcriptional reporter cell lines for other signalling pathways. For example, the Sapkota research group has also recently generated an endogenous 2G transcriptional reporter cell line for the BMP signalling pathway (Hutchinson *et al.*, 2019). In this cell line, the dual-reporter cassette was inserted in-frame and downstream of the endogenous promoter region for the BMP-responsive target gene DNA-binding protein inhibitor (alternatively referred to as inhibitor of

DNA-binding 1 or inhibitor of differentiation 1) *ID-1* (Hollnagel *et al.*, 1999; Katagiri *et al.*, 2002; López-Rovira *et al.*, 2002; Ying *et al.*, 2003). ID-1 is a transcriptional regulator that interacts with and negatively regulates basic helix-loop-helix (bHLH) transcription factors (Benezra *et al.*, 1990; Ruzinova and Benezra, 2003). The stimulation of HaCaT cells with recombinant human BMP_2 enhanced the relative luciferase activity 1.5-fold compared to unstimulated control cells. Co-incubation with the BMP type I receptor kinase small-molecule inhibitor LDN193189 (Cuny *et al.*, 2008; Yu *et al.*, 2008) attenuated the induction of luciferase activity, indicating that the transcriptional reporter cell line is dependent on BMP stimulation.

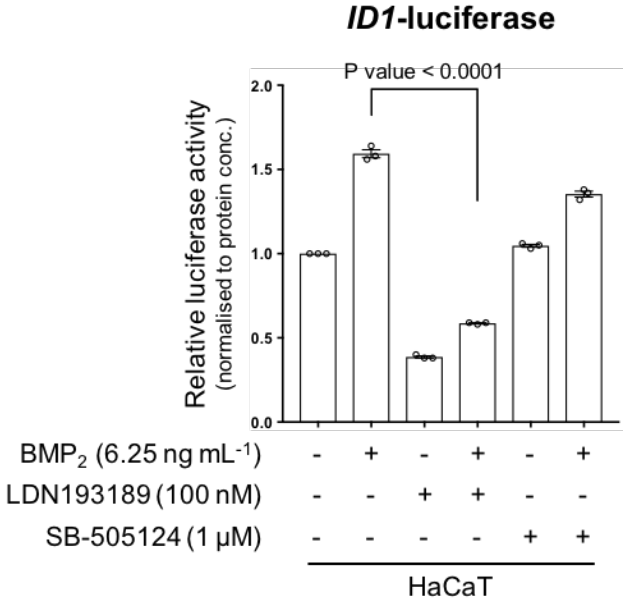


Figure 4A. The generation of an endogenous 2G BMP-dependent transcriptional reporter cell line

HaCaT 2G BMP transcriptional reporter cells were incubated with LDN193189 (100 nM), SB-505124 (1 µM) or an equivalent volume of DMSO and simultaneously stimulated with recombinant human BMP_2 (6.25 ng mL^{-1}) for 8 hours prior to cell lysis and luciferase assay analysis. The experiment was performed three times and the luminescence values were normalised to the total protein concentration of the respective cell lysate sample. Error bars represent the standard error of the mean (SEM).

In the data presented throughout this thesis, it has been demonstrated that three structurally unrelated small-molecule kinase inhibitors (MRT199665, bosutinib and dasatinib), that are known to potently inhibit SIK isoforms, all have the capability to modulate

TGFβ-mediated transcriptional responses. Furthermore, the effects of these kinase inhibitors occur at the same concentrations required to prevent the phosphorylation of the previously established physiological substrate of SIKs, the transcriptional coactivator CRTC3. The ability to demonstrate that an observed effect occurs with at least two structurally unrelated kinase inhibitors and at the appropriate inhibitor concentrations are both essential criteria for the use of pharmacological protein kinase inhibitors in the study of cellular signalling (Davies *et al.*, 2000; Cohen, 2009). Nevertheless, it remains conceivable that the modulatory effect on TGFβ signalling is occurring through inhibition of protein kinases other than the presumed targets of SIK isoforms. As such, one of the most rigorous experiments that can be conducted in order to ascertain that the observed effects of a pharmacological inhibitor are mediated via the inhibition of the presumed target kinase, and not by any 'off-target' inhibition, is to demonstrate that the effects of the inhibitor disappear when an inhibitor-resistant mutant of the protein kinase is expressed. As previously referred to in section 3.5.2, SIK isoforms are unique amongst the AMPK-related family of protein kinases in that they possess a threonine residue at the 'gatekeeper position' within their kinase domain. Threonine contains a relatively small amino acid side chain and as a consequence, HG-9-91-01 is selective for SIKs over other members of the AMPK-related family because it targets a hydrophobic pocket formed by the presence of the small threonine gatekeeper residue. Therefore, in principal, mutation of the gatekeeper threonine residue to an amino acid residue with a comparatively larger side chain would abrogate the ability of HG-9-91-01 to inhibit SIK isoforms. Indeed, research has elegantly demonstrated that the replacement of the gatekeeper threonine residue of SIK2 (Thr96) with a glutamine residue (T96Q) abolishes the ability of HG-9-91-01 to inhibit SIK2 *in vitro* (Clark *et al.*, 2012). Moreover, overexpression of SIK2^{T96Q} in macrophages prevented the ability of HG-9-91-01 to induce an anti-inflammatory macrophage phenotype characterised by enhanced levels of the anti-inflammatory cytokine IL-10 (Clark *et al.*, 2012). Although mutation of the gatekeeper threonine residue in SIK isoforms abolishes the inhibitory action of HG-9-91-01, it does not render them resistant to MRT199665. Subsequent research however demonstrated that SIK2^{T96Q} is resistant to both bosutinib and dasatinib (Ozanne, Prescott and Clark, 2015). As demonstrated in this thesis, both bosutinib and dasatinib can recapitulate the effects observed with the MRT199665 compound on TGFβ-mediated transcriptional responses. Consequently, investigating whether the expression of the SIK2^{T96Q} inhibitor-resistant mutant can prevent the attenuation of TGFβ-induced target

gene transcription caused by bosutinib and dasatinib would be a critical experiment by which to experimentally validate the role of SIKs in TGFβ signal transduction.

Although the precise molecular mechanisms by which SIKs modulate the TGFβ pathway remain to be elucidated, I hypothesise that SIKs may function at the level of transcriptional regulation. Since the discovery of SIK1 in 1999 (Wang *et al.*, 1999), followed by the identification of SIK2 and SIK3 in the early 2000s (Horike *et al.*, 2003), substantial progress has been made on illuminating the cellular functions of SIKs. Thus, it has become apparent that SIK isoforms have important regulatory functions in metabolism (Sakamoto, Bultot and Göransson, 2018) and the innate immune system, specifically macrophage polarisation (*i.e.* the interconversion of inflammatory and anti-inflammatory phenotypes of macrophages) (Clark *et al.*, 2012; MacKenzie *et al.*, 2013; Lombardi *et al.*, 2016b; Darling *et al.*, 2017). SIKs primarily function to regulate target gene expression through the modulation of transcriptional co-regulators.

At present, the most well-characterised physiological SIK substrates are the transcriptional coregulators CREB-regulated transcription coactivators (CRTCs) (Altarejos and Montminy, 2011; Clark, 2014; Sakamoto, Bultot and Göransson, 2018) and class IIa histone deacetylases (HDACs) 4 and 5 (Berdeaux *et al.*, 2007; Mihaylova *et al.*, 2011; Wang *et al.*, 2011; Patel *et al.*, 2014; Henriksson *et al.*, 2015; Sakamoto, Bultot and Göransson, 2018). Phosphorylated SMAD3 has been reported to interact with and recruit the class IIa HDACs HDAC4 and HDAC5 to mediate transcriptional repression (Kang *et al.*, 2005; Massagué, Seoane and Wotton, 2005; Ross and Hill, 2008; Hill, 2016). Therefore, it is conceivable that the role of SIKs in modulating TGFβ-mediated transcriptional responses is occurring through the regulation of activated SMAD3-HDAC4/HDAC5 transcriptional complexes. One potential method by which to investigate this possibility is by assessing whether the small-molecule inhibition of SIK kinases affects the interaction of SMAD3 with HDACs via co-immunoprecipitation analysis or whether the histone acetylation status of target gene promoter regions is affected by performing chromatin immunoprecipitation experiments using antibodies that recognise acetylated histone H3 or H4 followed by RT-qPCR analysis using primers that amplify specific promoter sequences. Although the observations reported in this thesis project identify a novel function for SIKs in the regulation of TGFβ-mediated transcriptional responses, previous research has implicated a role for the SIK1 isoform in the TGFβ signalling pathway. Kowanetz *et al* reported that SIK1 is a direct transcriptional target

of SMAD-dependent TGFβ signalling and that upon induction, interacts with the inhibitory SMAD7 and functions to down-regulate the activated TGFβ type I receptor kinase (Kowanetz *et al.*, 2008). Consistent with the data presented in this thesis whereby small-molecule kinase inhibition of SIK isoforms enhances the transcriptional induction of the CDKIs $p21^{CIP1}$ and $p27^{KIP1}$ in response to TGFβ stimulation, Kowanetz *et al* observed that the TGFβ-induced upregulation of $p21^{CIP1}$ and $p15^{INK4B}$ is enhanced upon siRNA-mediated knockdown of SIK1. However, contrary to the data presented in this thesis, depletion of SIK1 also resulted in the enhancement of TGFβ-induced *PAI-1* and *SMAD7* mRNA expression (Kowanetz *et al.*, 2008). This suggests that the precise nature of SIK function in the TGFβ signalling pathway may be dependent on cellular context and therefore further investigation is required in order to ascertain how SIKs regulate TGFβ signalling in different contexts.

In attempting to elucidate the mechanism by which SIKs regulate TGFβ signalling, one of the most intriguing observations made during the course of this thesis project is that SIK2, and to a lesser extent SIK3, are able to robustly phosphorylate SMAD3 *in vitro*. It is possible that SIK-mediated phosphorylation of SMAD3 may function to regulate the interaction of SMAD3 with DNA promoter sequences or transcriptional regulatory partners. As a result, subsequent research will concentrate on using mass spectrometry-based approaches to identify the potential SIK-dependent phosphorylation sites on SMAD3 and investigate the functional and physiological relevance of these phosphorylation sites.

In addition to determining the molecular target of SIKs in the context of TGFβ signalling, one of the critical outstanding questions that remains to be addressed is the extent to which SIKs may regulate TGFβ-mediated transcriptional responses. Are SIKs involved in the regulation of a specific subset of TGFβ target genes, or do they regulate the global TGFβ-dependent transcriptome? We sought to address this question by performing RNA sequencing (RNA-seq) using wild type MEFs and two independent SIK2/SIK3 mutant MEF cell lines. However, the data obtained from this analysis revealed a substantial degree of variation in the gene expression profiles of the basal unstimulated states between each of the three MEF cell lines such that it was challenging to generate any meaningful conclusions. The transcriptomic differences observed between these MEF cell lines may have been a consequence of the immortalisation procedure. Primary cell lines are of significant value in research because they have not been extensively passaged in culture and thus possess the same genetic composition as the animal from which they were derived. However, the major

limitation of primary cells is that they can only be passaged a limited number of times in culture before they undergo growth arrest and cellular senescence. In order to circumvent this limitation, primary cells can be subjected to cellular transformation via the retroviral-mediated transduction of the SV-40 large T antigen, which then allows the cells to be propagated in culture indefinitely. However, cellular transformation using the SV-40 large T antigen is known to perturb multiple cellular signalling pathways (Ahuja, Sáenz-Robles and Pipas, 2005) and it is plausible that this has contributed to the transcriptome variations observed in the MEF cell lines used for the RNA-seq analysis. Moreover, one of the principal disadvantages of using primary cell cultures is the possibility that they may consist of multiple different cell types, a factor which may also contribute to the differences observed in the transcriptome of the three MEF cell lines. Therefore, an alternative approach using SIK inhibitors in cultured human cancer cell lines may be a possibility in any future transcriptomic analysis experiments, although this approach would inevitably possess considerable limitations such as kinase inhibitor selectivity. Moreover, the generation of SIK isoform knock-out (KO) cell lines using CRISPR-Cas9 would be an incredibly valuable tool in which to analyse the effect of SIKs on the TGFβ-dependent transcriptome.

One of the key findings arising from this thesis project is that the inhibition of SIKs using either one of the small-molecule kinase inhibitors MRT199665 or bosutinib is capable of potentiating the TGFβ-mediated apoptotic cell death of NMuMG cells. The ability of TGFβ to induce apoptosis is considered to be one of the crucial mechanisms by which TGFβ functions as a tumour suppressor (Massagué, Blain and Lo, 2000; Bierie and Moses, 2006; Massagué, 2008b; Inman, 2011b). If the observation that SIK inhibition results in the potentiation of TGFβ-induced apoptosis can be substantiated in a cancer cell model, this raises a novel and interesting therapeutic potential. The majority of pharmacological interventions targeting the TGFβ pathway have focused upon inhibiting the pathway, for example by using anti-sense oligonucleotides or antibodies directed against TGFβ ligands or small-molecule kinase inhibitors of the TGFβ type I receptor (Akhurst and Hata, 2012; Akhurst, 2017). However, these strategies may be hindered by the potential of impeding the tumour suppressive effects of TGFβ in addition to disrupting its numerous homeostatic functions. Hence, targeting specific facets of the pathway as opposed to the complete inhibition may be a more advantageous therapeutic approach. Consequently, in specific tumour types which depend on TGFβ signalling, SIK inhibition may be a potential therapeutic approach by which

to promote apoptotic cell death of those tumour cells. Previous published research, along with research conducted during the course of this thesis project, has demonstrated that the clinically approved small-molecule inhibitors bosutinib and dasatinib are potent inhibitors of SIK isoforms (Sundberg *et al.*, 2014; Ozanne, Prescott and Clark, 2015). Both bosutinib and dasatinib have been successfully used for the treatment of patients with imatinib-resistant or imatinib intolerant chronic phase Ph+ CML or ALL, therefore this indicates that inhibiting SIKs for therapeutic purposes can be tolerated in patients.

Overall, the experiments presented throughout this thesis project have provided evidence for the identification of the protein kinases SIKs as novel regulators of the TGFβ signalling pathway. Although the precise molecular mechanisms underlying this finding remain to be elucidated, the data indicates that SIKs are an additional regulatory component of TGFβ signalling and the results presented may contribute toward the further understanding regarding the contextual determinants of TGFβ signalling in normal and pathological conditions.

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